In Memoriam Prof. María Rosa Miracle, Limnologist

Limnetica Special Issue (July 2022)

Edited by A. Camacho, E. Vicente, J.M. Soria, H. Dumont & S.S.S. Sarma

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PREFACE

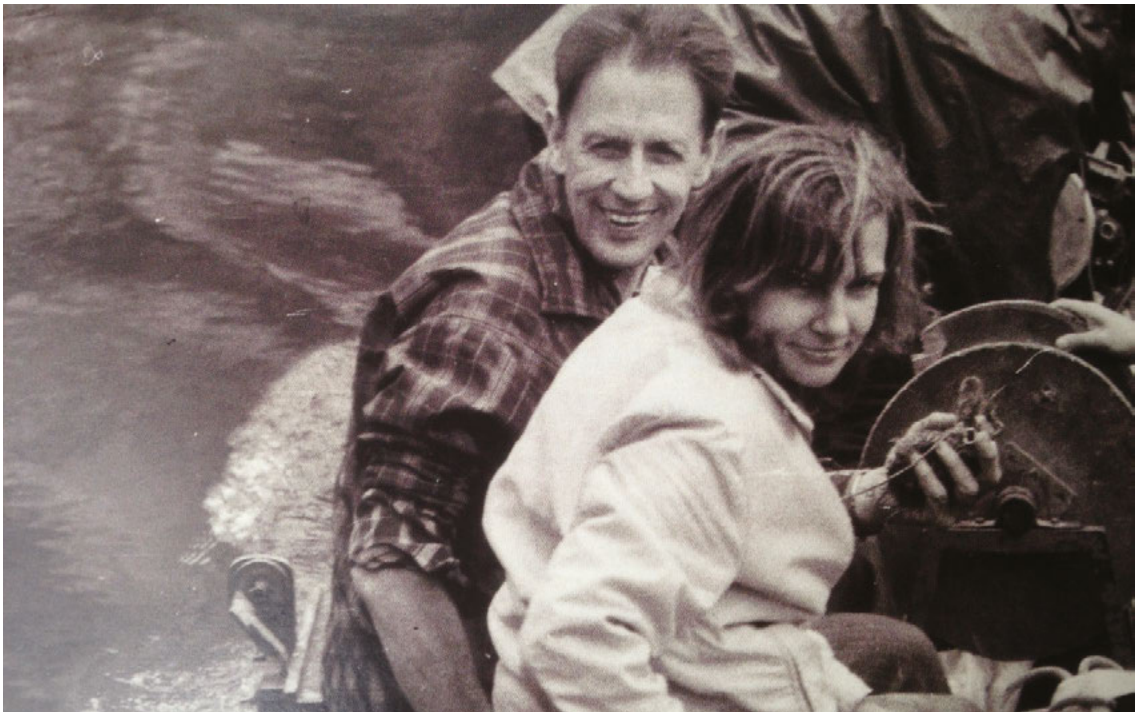
This is a book in memoriam to Professor Maria Rosa Miracle. Collecting 42 invited manuscripts in tribute to Prof. Maria Rosa Miracle. She passed away on 28th May 2017 in Valencia (Spain), at the age of 72, when she was still active as Emeritus Professor at the University of Valencia. During her nearly five decades of dedication to research and teaching, she became globally a highly influential limnologist. Her lines of research in limnology paved the way for many new interdisciplinary themes, some of which can be found in this special volume.

Soon after Maria Rosa passed away, the Iberian Association of Limnology (AIL) decided to dedicate, in her honour, a special issue of *Limnetica*, the indexed journal of the Association to which she was always associated as Author, Reviewer or Editor. Most of the present collection of papers was published in *Limnetica* in homage to Maria Rosa. Manuscripts have been contributed by various invited authors, many of whom seek answers to some of the puzzling limnological questions raised by Maria Rosa, and on the themes she liked to work on. Many of the articles have been elaborated by her former students, colleagues and collaborators, trying to address some of the unfinished products of her last few months of research activity. While collaborating with different colleagues, she left a wealth of research data which will perhaps be published in near future following her ideas and the guidelines that she has left behind.

This preface also contains yet published some personal reflections from some limnologists who knew or have closely worked with Prof. Miracle, some even for more than forty years. These give us a vision of Maria's friendly relationships with other limnologists from the beginning of her research to the latest global scientific projects.

Valencia, 28th May, 2022

Guest Editors of the Special Volume
A. Camacho, E. Vicente, J.M. Soria, H. Dumont and S.S.S. Sarma,



Prof. Margalef and Maria Rosa on the boat at Lake Banyoles. Ca. 1970.

Claudia Ricci, Dr. Edmondson with Maria Rosa and Lynda May in Gargnano during the Rotifer Symposium. 1988.

A tribute to Maria Rosa Miracle

Eduardo Vicente and Antonio Camacho
Department of Microbiology and Ecology & Cavanilles Institute for Biodiversity and Evolutionary Biology. University of Valencia, Spain.

Professor Maria Rosa Miracle, limnologist. In memoriam

Maria Rosa Miracle was born on 2nd June 1945 in Barcelona. She studied at the University of Barcelona where she was awarded with her BSc (honours, 1968) and PhD (*cum laude*, 1974) degrees. As a former PhD student of Prof. Dr. Ramón Margalef, she conducted field work on Lake Banyoles (Girona, Spain) studying for several years the seasonal succession, distribution and patchiness of zooplankton. After completion of her PhD dissertation, she spent a two-year research stay at the University of California, Davis, with Dr. Charles Goldman as supervisor.

Upon returning to Spain, she spent several years, until 1979, at the University of Barcelona as Associate Professor, developing mainly two projects financed by Research Scholar Government Public Works: the “Study on Pyrenean Lakes” and a “Study on the Spanish Mediterranean coastal lagoons”. In 1979 she went to the Department of Zoology, Oregon State University, Corvallis, invited by Dr. Charles King, to participate in the project “Rotifer population dynamics, experimental laboratory studies on survival”. At the end of 1979 she joined the University of Valencia as Senior Professor of Ecology, setting up the Limnology Research team there, and occupied the prestigious *Chair on Ecology* in 1981, following her academic activity until her retirement by September 2015. Thereafter, she continued as Emeritus Professor at the University of Valencia.

For more than forty years, Maria Rosa Miracle was a tireless and unflagging worker, completely devoted to research and teaching activities. She served as the Head of the Ecology Department at the University of Valencia between 1981-1987 and 2003-2010, as the President of the Academy of Environ-mental Biology, India from 1987 to 1990, and as the President of the Iberian Limnological Association from 1994 to 2002. Maria Rosa was also Associated Editor of five international scientific journals.

Prof. Miracle supervised more than 20 PhD students and many of them are now university professors, including three currently Chair Professors on Ecology. In addition, three of former PhD students are now scientists working at research centers in Germany, one in France and one more at Colegio de la Frontera Sur in Mexico. Also, three of them teach at secondary schools, and others work as environmental managers on public or private entities. She also supervised more than 20 Master theses. Prof. Miracle had a passion for teaching and was revered by her students.

Maria Rosa was Principal Investigator or participant in over 20 highly competitive Spanish funded research projects, over 10 European or International Projects, and 18 technical studies and reports. She authored or co-authored more than 200 indexed scientific articles, 21 books or book-chapters, and organized 16 International Congresses or Workshops. She also authored over 250 Abstracts or Extended Abstracts in Congresses and Symposia.

This was just a numerical summary of forty years of professional activities. In addition to study many of Spanish lakes (mainly karst lakes), wetlands and lagoons, she also visited and studied aquatic ecosystems in many other countries, collaborating with some of the most reputed limnologists around the world. Among others, she shared research with Prof. Henri Dumont (Ghent University, Belgium), actively participating in a limnological expedition to the Algerian and Tunisian Sahara in 1976, as well as in a study of primary production in marginal lakes associated with River Niger in 1993.

She performed several research stays at the Windermere Laboratory (Freshwater Biological Association, UK) with Dr. Bland Finlay, to study the microaerophilic and anoxic environments in 1983, and also conducted some electron microscopy studies of ciliates and bacteria at the oxic-anoxic interface of strat-ified lakes in 1992.

She also conducted joint research with Prof. Peter Tyler on the interfaces and chemo-clines in the stratified marginal lakes of Gordon river in Tasmania in 1987, and pursued in the Protistolo-gy Laboratory, Deakin University (Warrnambool, Australia), to perform a Limnological study of stratified deep areas in the Hopkins estuary. With Prof. Wayne Wurtsbaugh, she performed a research stay in the Utah State University, Logan, USA, studying the eutrophication of the Farmington basin in the Great Salt Lake in 2002. With Dr. Keve Kiss, of the Hungarian Academy of Sciences, she developed an Spain-Hungary integrated project for plankton studies from 2005 to 2007. Most recently, with Dr. Victor Alekseev of the Russian Academy of Sciences, she jointly lead a study on the taxonomy of the cyclopoid copepods of confusing identification by comparison with specimens from the type locality in 2011, and also conducted a study of microcrustaceans at the shoreline of the Lake Baikal and wetlands near Irkutsk in 2012.

Similarly, reputed senior scientists have visited Maria Rosa's laboratory to study, among others, the plankton of Albufera de Valencia and other shallow lakes in the Mediterranean coast, including many of the above-mentioned co-workers but also Prof. Brian Moss (UK), Prof. Manuel Elías (Yucatan-Mexico) and Profs. S.S.S. Sarma and Nandini Sarma (UNAM-Mexico). Another limnological area of her interest was the stratified lakes near Arcas, doing a common project with Dr. Finlay, as well as the karst area of river Guadazaon polje, conducting a project with Prof. Wurtsbaugh in both Lake El Tejo, and in the internationally well-known meromictic Lake La Cruz (both systems in Cuenca, Spain), that was also visited several times by her with other senior scientists such as Dr. Jakob Zopfi and Prof. Raymond Cox in addition to those already mentioned as collaborators.

All her scientific and academic achievements were only possible through her lively and passionate attitude towards the challenges of the new, the uncommon and the unknown, with a desire and disposition to increase knowledge on extreme or rare environments wherever they might be encountered, seeking those inland waters, lakes, wetlands in Europe, Eastern Asia, America or Africa even getting to the barely reachable Saharan or fluvio-marginal lacustrine waters at the Niger Delta.

The incorporation of Professor Miracle as faculty member of the University of Valencia led the start-ing point of the Limnology school at this University, which over the years became the second relevant school in this area, inspired by that initiated by Professor Dr. Ramón Margalef at the University of Barcelona, of which Dr. Miracle was part, fifty years ago, as a PhD student. The impact of the limnolo-gists trained in the research team of Prof. Miracle is reflected in the academic, scientific and professional development of the PhD students trained under her supervision.

In addition to her achievements in Academia, the most remarkable feature about her character is that she was a hard worker and a very good and gentle person. Maria Rosa was all her life a great supporter of Spanish Limnology and will be missed by her colleagues and all those who interacted with her. Being a very shy person, science was her life, but her two sons, Dicky and Dani, and her daughter, Olga, were her love. Those that valuated her as a person and as a scientist will miss Maria Rosa.

Claudia Ricci
University of Milan, Italy.

Maria Rosa Miracle, the Rotifers and I: a friendship story across Rotifer symposia

I met Maria Rosa Miracle at the first Rotifer Symposium, held in Lunz am See at the Biological Station of the Austrian Academy of Science. At that time, September 1976, both Maria Rosa and I were quite young, but while I was just a beginner in my studies on rotifers, Maria Rosa had already finished her PhD on monogonont rotifers under the supervision of Ramon Margalef, one of the brilliant ecologists of the last century. At Lunz, we shared a room at a 'bed and breakfast' not far from the Biological Station; this gave us an excellent opportunity to know each other. Maria Rosa was quite shy and did not talk much until she could feel comfortable: shortly we became friends. One of our common traits was to be late-comer, and often we risked to arrive late at the morning scientific sessions at the Biological Station. Hopefully, almost every day Alois Herzig saved us: he drove every morning to the Station and saved us from impolite delays at the meeting.

At Lunz meeting, Maria Rosa was already an experienced scientist: she was invited to give a review presentation on distribution and migration of planktonic rotifers. The whole audience appreciated the lecture: her talk was excellent, revealing a bright scientist, specialist in systematics, biology and ecology of monogonont rotifers.

Maria Rosa attended most Rotifer Symposia, she missed three of them: the ones hold in Belgium (1979), Austria (2003), and Germany (2009). Nevertheless, the results of a collaboration between Maria Rosa and Charles King were presented at second rotifer meeting, in Gent (Belgium). Compensating her absence at Gent symposium, she was very busy at the third one (Uppsala, 1983), where she arrived with a colleague (Eduardo Vicente) and a young PhD student (Manuel Serra): both are now well-known ecologists.

During June 1991, she organized the sixth rotifer symposium in a Catalan village, Banyoles, not too far from Barcelona. Adjacent to the village is a lake, Banyoles, well-known to Maria Rosa because there she had been studying zooplankton composition and movements for her PhD thesis. Year 1991 was a very busy time for Spain because of the Olympics scheduled for the following year and organized in Catalunya: even Barcelona airport was under restoration, complicating the participants' movements. On the other hand, village and Lake Banyoles were going to host the water competitions of the Olympic Games. Thus, most streets to enter and cross Banyoles village were either interrupted or deviated. Maria Rosa was so used to move around in Banyoles that she did not realize that a bus is not a car and cannot drive along narrow cross roads. As a consequence, the bus got stuck at a cross and we were suggested to walk to the monastery of Sant Esteve, place of our lodging and of the scientific meeting. Maria Rosa was so familiar with Banyoles and surroundings that, in occasion of the excursion, she and Eduardo got 'lost' while the entire group of rotiferologists was sitting in the bus waiting for them: that was the first case of 'lost organizers'. As a matter of fact, any occasion was good for Maria Rosa to water samplings and rotifer collections. As it was pointed out, no need for panic: they knew the language and could ask for instructions; actually, they did not need instructions, only time to sample.

Both Maria Rosa and I were quite regular at Rotifer Meetings, and we enjoyed the opportunities to get together on several occasions, also outside the context of the Rotifer symposia. One of the best opportunities we had was the participation in the committee to evaluate a PhD dissertation at the University of Sevilla in 1989. Ramon Margalef headed the commission. At the end of the academic duties, the whole commission was kindly invited to visit the famous *Coto de* Doñana; this provided us a unique opportunity to enjoy that beautiful and unique habitat commented by two unusual 'guides', Ramon Margalef and Francisco Garcia Novo, head of the Biology Department and a well-known plant ecologist.

During that same year, 1989, Maria Rosa came to Milan and visited our Department of Biology: after spending a few days together in the laboratory, we moved to Bormio, a touristic village high on the Italian Alps to attend a meeting of the Society of Italian Zoologists (UZI), scheduled to be held there. Ramon Margalef, too, was attending the same meeting and Maria Rosa and I could spend some time with him and his wife.

On July 1992, invited by Maria Rosa, I participated in a PhD committee at Valencia University. After the academic duties, Maria Rosa and Eduardo Vicente invited me to join a sampling campaign in the surroundings of the beautiful town of Cuenca where they had to go sampling. Although on a more touristic mood, I was happy to join them and Maria Rosa's daughter, Olga, joined the group; we all moved by car. On the first day, when night came, we were quite far from any touristic place, and neither hostel nor B&B nor hotel was around. In absence of alternatives, Maria Rosa and Eduardo decided that we could sleep in the car..., we were still 'young', then. The rest of our sampling campaign was very exciting and, for sure, more comfortable.

At the beginning of 2000, an international group of scientists studying parthenogenetic organisms built a scientific network (PARTNER = parthenogenetic organisms) the aim of which was to evidence and compare the traits of the many organisms (both animals and plants) that reproduce parthenogenetically. One of the meetings was held in Valencia, organized by Manuel Serra, Maria Rosa and their lab group. Maria Rosa attended the entire meeting, nevertheless, together with Eduardo could find the time to take me and my husband to visit unique beautiful places in the surroundings of Valencia, such as 'la Albufera', i.e. salt marshes and lagoons, that host peculiar and resistant inhabitants including many rotifer species.

Although our investigation fields differed quite much, synecology of monogonont rotifers by Maria Rosa and autecology of bdelloids by me, our scientific discussions were always interesting and fruitful to me. Our friendship, sometime with short contacts and occasional encounters, lasted until the last Rotifer meeting (Czech Republic, 2015) and later on, although with a few email messages. In fact, Maria Rosa never addressed any aspect of her private life with colleagues and the same was with me, as from my personal experience. The fact that she was sick was unknown to most of us and her death for most of us, if not everybody, totally unprepared to listen such unpleasant and sad news.

Our last encounter was in 2015 at the last Rotifer meeting in Czech Rep. During the year 2018, another Rotifer Meeting had taken place in USA: everybody would have missed Maria Rosa Miracle. Maria Rosa's and my research focused on rotifer biology and ecology, but dealing with different animal groups (monogononts for her, bdelloids for me) and under different points of view. In spite of our personal and professional differences, our friendship lasted for 40 years, sometimes with long silences, but with reciprocal pleasure at every meeting occasion; together with the entire rotiferologist community I shall miss her.

Ramesh D. Gulati **+**
Netherlands Institute of Ecology, Wageningen, The Netherlands.

Maria Rosa Miracle, a Tribute

A lot has already been said and written about Maria Rosa Miracle since her tragic death on 28th May, 2017. I will restrict my writing about Maria Rosa’s passing away to my personal meetings with her, especially since I came to know her more personally in the early 1990s. My first personal contact with Maria Rosa was in 1991 when I visited Banyoles, Spain, to participate in the deliberations of the 6th Rotifer Symposium that she organized there. Of course, I met Maria Rosa on a couple of occasions earlier but then I did not realize how important the rotifers were to the understanding of their role in the food-chain of aquatic organisms. This was also partly because I was not interested in Rotifera as an important group of zooplankton. Therefore, the Congress in Banyoles was for me an eye opener in many respects concerning the importance of rotifers as zooplankters as well as their origin.

I had several opportunities at the Rotifer Meeting in 2015 in Ceske Budejovice, Czech Republic, to meet Maria Rosa and discuss with her some issues concerning Rotifers. I learnt a lot from this meeting with her. It was around this period that I had been also editing a Springer Book on the Ecology of Meromictic Lakes (Ecological Studies 228; Eds. Gulati *et al.*, 2017). Maria Rosa Miracle and her colleagues contributed the Chapter 8 on "Lake La Cruz, an Iron-Rich Karstic Meromictic Lake in Central Spain". Their book chapter was of great interest to me as far as the importance of microorganisms in meromictic lakes. I vividly recall my useful discussions with Maria Rosa both on rotifers and microorganisms in such lakes.

A part of my contacts and intense interest in the works and well-being of Maria Rosa was undoubtedly due to the feed backs from my friends Nandini Sarma and SSS Sarma, in Mexico. They were great friends of Maria Rosa and worked with her at Valencia on various Spanish lakes. At the invitation of the Organizers, I was actually in Mexico, early in 2017, attending the International Shallow Lakes Meeting at Merida, Mexico, where I learnt about Maria Rosa's deteriorating health condition, although she was already seriously sick. We were also well kept informed about her health condition by Eduardo Vicente, a senior Spanish colleague of Maria Rosa who too was in Mexico attending the same Meeting at Merida. The expected happened and Maria Rosa passed away (peacefully) in the end of May 2017. It was, indeed, a very sad and tragic event but we have to accept it.

Ramesh Chandra Dalela
Editor-in-chief Journal of Environmental Biology *&*
Retired Professor at D.A.V. College, Muzaffarnagar, U.P. State, India.
Founder President of the Academy of Environmental Biology (India)

Prof. Maria Rosa Miracle has been an epitome of dedication and professionalism. As a teacher and researcher, she left everlasting impression in the form of work culture, ethos and discipline which will always be remembered by her students, fellow researchers and friends. Prof. Maria Rosa was closely associated with the Academy of Environmental Biology (India). She first served as Vice President of the Academy of Environmental Biology (AEB) during 1985-1987 and then as President of the Academy (AEB) for the period 1987-1990. During this period, she visited the Academy's headquarter and stayed with Dr. R. C. Dalela for about a month, visited a few places with him and delivered lectures besides presiding over the Annual Session of the AEB. Dr. Dalela fondly remembers the pleasant days that he and Mrs. Dalela spent with Professor Maria and her daughter at Muzaffarnagar, India. She also served as one of the members of Editorial Board of *Journal of Environmental Biology* for some time during 90's and was instrumental in raising the quality and standard of the Journal by her critical reviews and suggestions from time to time.

Dr. Dalela and Mrs. Dalela along with the Academy (AEB) and Journal (JEB) family wish the departed soul an eternal peace and her family the strength to bear this irreparable loss.

Singaraju S.S. Sarma
National Autonomous University of Mexico (UNAM)

Prof. Maria Rosa Miracle achieved international recognition and as a result was asked to serve in leader-ship roles outside of her native country. Beginning in the early 1980s she was closely associated with Academy of Environmental Biology (India) (AEB). She served as Vice-President of the Academyduring 1985-1987 and then became President for the period 1987-1990. During this time, she visited the Academy and contributed to the proceedings edited by R.C. Dalela, S. Kant, and S. Vahra (1988). In fact, the Editor-in-Chief of JEB, Dr. R.C. Dalela fondly remembers the pleasant days that he spent with Prof. Maria Rosa and her daughter together with Prof. Eduardo Vicente. Prof. Maria Rosa with her students also published a key article on the effects of insecticides on ecotypes of the rotifer *Brachionus plicatilis* (JEB; Vol. 7(4): 259-275, 1986). This paper is still being cited (Mills *et al.*, 2017 *Hydrobiologia* 796(1): 39-58). This important research was one of the first to show that different ecotypes of *B. plicatilis* respond differentially when subject to environmental stress. We now know that *B. plicatilis* is actually a cryptic species complex consisting of as many as 15 species (Mills *et al.*, 2017). Thus honour-ing her by dedicating this special volume is a token to express our gratitude for her services and contri-bution, not only for the AEB, but also for the entire community of limnologists.

My association with Prof. Maria Rosa dates to 1991 when she organized the prestigious 6th International Rotifer Symposium, which was held in Banyoles (Spain). It was only possible for me and other young scientists from developing nations to attend this meeting because Prof. Maria Rosa waved all costs associated with my participation. Since the late 1990s, my wife, Prof. S. Nandini, I met Prof. Maria Rosa in many international events such as Symposia on Rotifera, Cladocera, Copepoda, SIL, etc. Our research collaboration grew strongly in recent years. For example, during last five consecutive summers, we carried out various experiments on zooplankton feeding ecology, predation, and ecotoxicology at her laboratory. This productive collaboration has already resulted in several publications with a few more in review or in preparation for submission. Prof. Maria Rosa was also keen to deliver a key note address on Spanish hypertrophic lagoon, Albufera of Valencia at the 9th Shallow Lakes Meeting in February 2017 (Merida, Mexico). Unfortunately, by this time her health began to deteriorate considerably and thus she could not attend the meeting, although Prof. Eduardo Vicente delivered the invited talk of their joint work on Albufera at this meeting. As a researcher, Prof. Maria Rosa's contributions are numerous; as a mentor to her students she was indispensable; as a friend she was a delight to be with.

Picture Gallery

Maria Rosa Miracle and Claudia Ricci during First Rotifer Symposium, Lunz 1976.

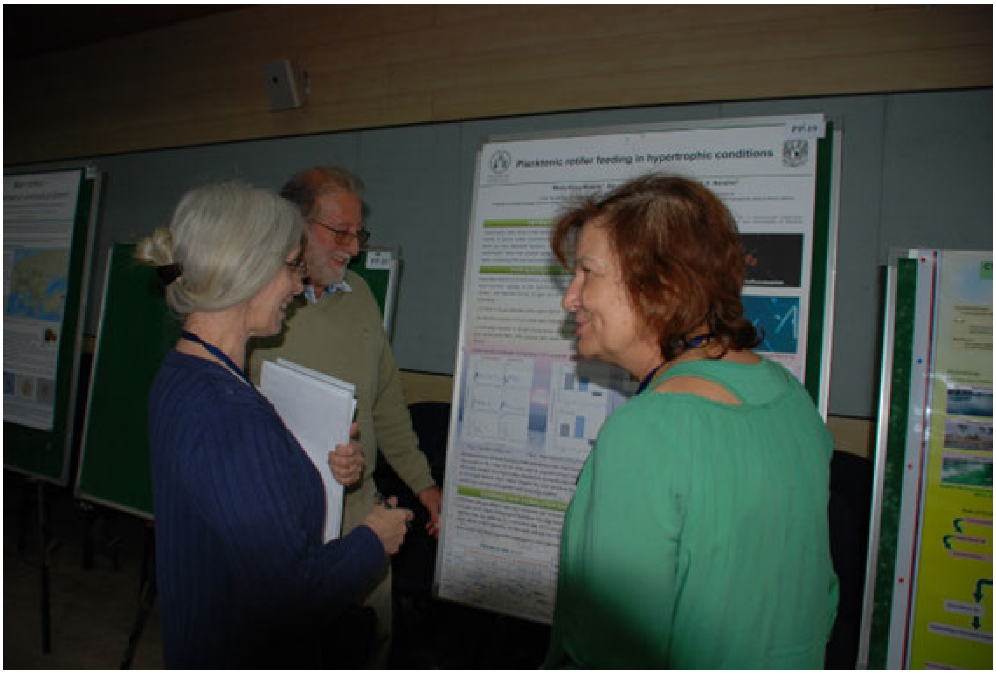
Verona Roman amphitheatre. Excursion during V Rotifer Symposium, 1988

Field trip with students at Escaba stream (Tejadillos, Cuenca), 1991.

Prof. Miracle with her limnology team during First Iberian Congress of Limnology (Evora, Portugal 1998).

Picture group after Limnology Congress dinner (Valencia, Spain, 2000).

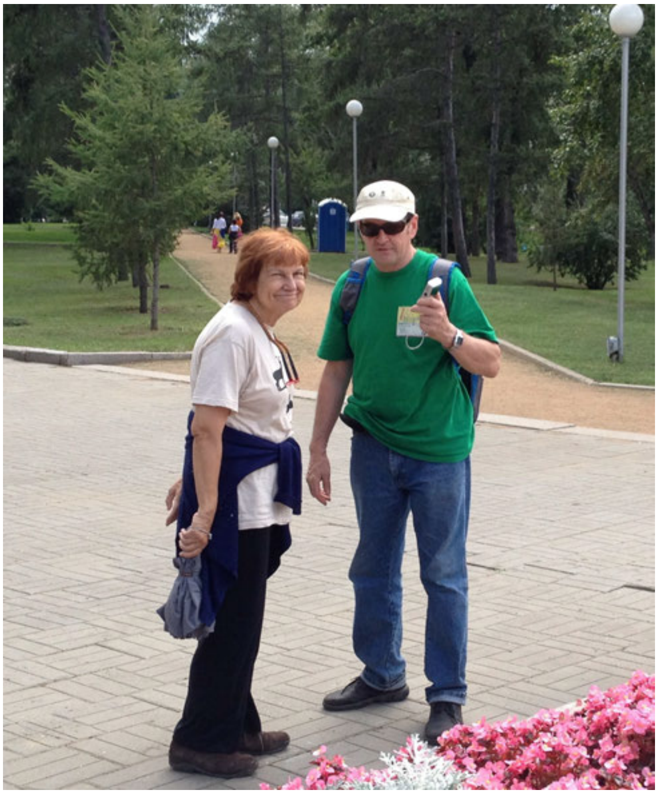
Great Salt Lake: Maria Rosa is sampling zooplankton in Great Salt Lake (USA). 2002.



Linda May, E. Vicente and Prof. Miracle duringXIII Rotifer Symposium, Shillong, India 2012.

Sampling in a pond in the tea groves region (Meghalaya State, NE India, 2012).

Sampling and collecting plankton in Lake Baikal (Siberia, 2012)



Victor Alekseev and Prof. Miracle, Irkutsk Historical Garden 2012.

Juliana Dias, Prof. Miracle and E. Vicente at the Department seminar (University of Valencia, 2012).

Next to a doline after sampling Lake La Cruz. From right to left, Prof. Raymond Cox, Jennifer Thompson, Dr. Amelia Rotaru, Javier Soria, Prof. Miracle, Dr. Nicole Posth and Dr. Simon Poulton. 2014.

Danish research group after one week sampling at Lake La Cruz at the stairs of Cuenca Cathedral, 2014.

Iberian Congress of Limnology dinner in Santander, 2014.

Prof. Miracle, Mariloli Sendra and E. Vicente sampling in Llauset reservoir, 2015.

PhD graduation party of Gabriela Onandia, Valencia, 2015.

Prof. Miracle sampling in small ponds of Valencia , 2015.

Prof. Miracle, Luigi Naselli-Flores, Emanuela and Maria Sahuquillo in ricefields of Albufera, 2016.

Marcelo Pompeo, Prof. Miracle, Viviane Moschini and E. Vicente, Valencia, 2016.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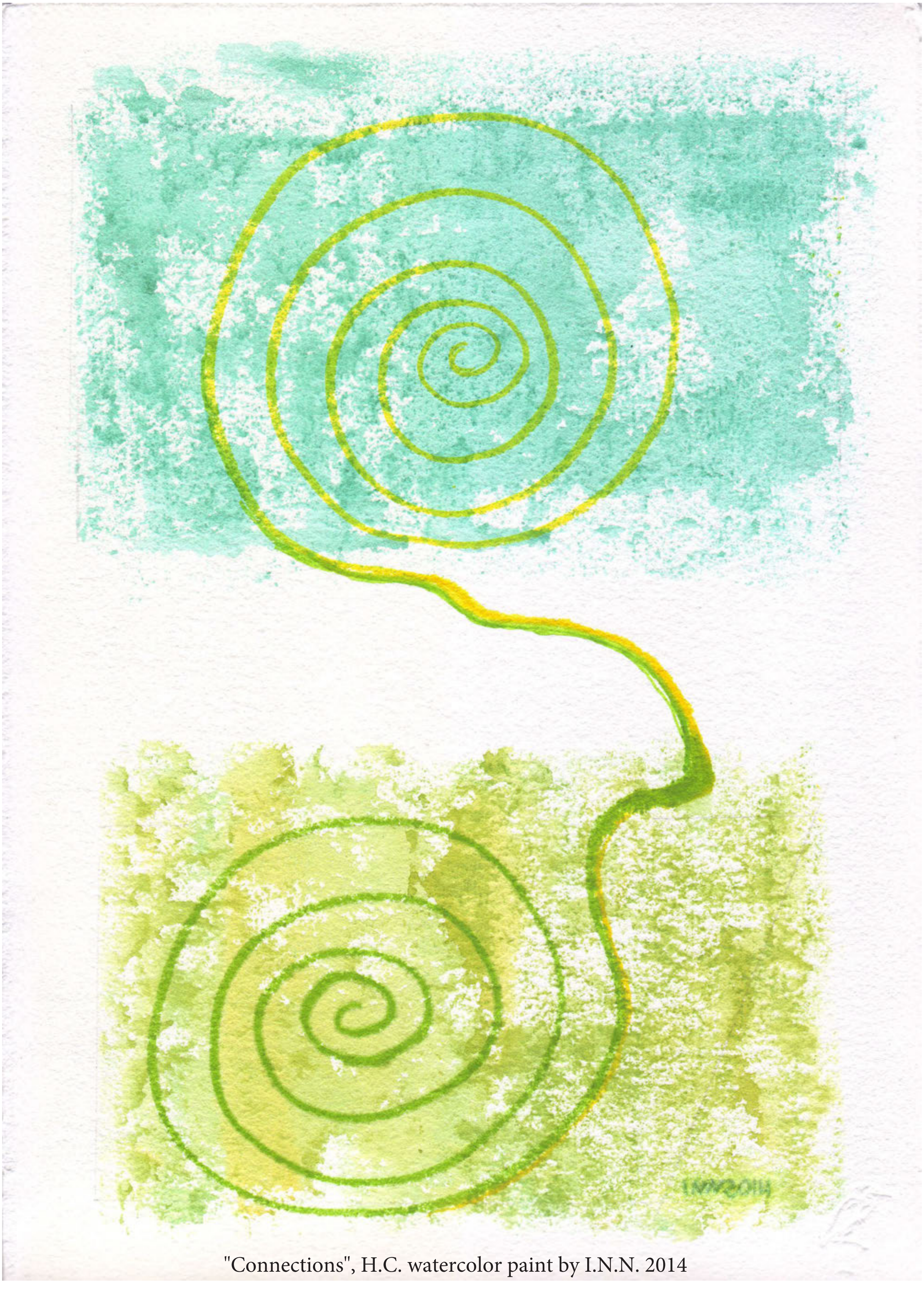

"Connections", H.C. watercolor paint by I.N.N. 2014

Meromictic lakes, phytoplankton ecology

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Temporal succession, spatial dynamics and diversity of phytoplankton in the meromictic Laguna de la Cruz: the role of stratification patterns

Sendra, M. D., M. R. Miracle, E. Vicente, A. Picazo and A. Camacho*

Instituto Cavanilles de Biodiversidad y Biología Evolutiva. Universidad de Valencia. E–46980 Paterna, Valencia. España.

* Corresponding author: antonio.camacho@uv.es

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ABSTRACT

Temporal succession, spatial dynamics and diversity of phytoplankton in the meromictic Laguna de la Cruz: the role of stratification patterns

Laguna de La Cruz is a small meromictic karstic lake located in Cuenca (Central Spain), whose stratification and thermal mixing patterns largely determine the structure and composition of its phytoplankton populations. In this lake, the succession in the oxic zone of the water column starts, with the autumnal mixing, with some diatom species, such as *Cyclotella delicatula*, of the functional codon **B**, dominating the community throughout the oxic water column during the winter. This species persists in spring, but then linked to deeper layers. In spring, at the beginning of the thermal stratification, picocyanobacteria, cryptophytes, and dinoflagellates develop in upper layers, corresponding, respectively, to the functional coda **Z**, **Y**, and **Lo**, with the first two being progressively restricted to the oxycline as the stratification advances. As stratification advances, dense populations of chlorophytes belonging to the functional groups **F**, **X**, and **J** develop in the epilimnion and the metalimnion, while towards the end of the summer *Cosmarium* species, belonging to the functional group $\mathbf{N_A}$, are selected and become dominant until the autumnal mixing favors again the diatoms. As a whole, the strong stratification of the water column allows for the appearance of different niches with a differential vertical distribution of the phytoplankton during the stratification periods, which favors its diversity within the whole water column. However, several types of disturbances, such as episodes of heavy rainfall, horizontal movements of water bodies and, in particular, the whitening that occurs every year towards the middle of summer, alter the successional pattern to some extent.

Key words: Phytoplankton, succession, functional groups, stratification, meromictic lakes

RESUMEN

Sucesión temporal, dinámica espacial y diversidad del fitoplancton en una laguna meromíctica, la Laguna de la Cruz: el papel de los patrones de estratificación

La Laguna de La Cruz es un pequeño lago cártisco meromíctico situado en Cuenca (Centro de España), cuyos patrones de estratificación y mezcla térmica determinan en gran medida la estructura y composición de sus poblaciones de fitoplancton. En este lago la sucesión en la zona óxica de la columna de agua se inicia con la mezcla otoñal, tomando protagonismo las diatomeas, con algunas especies como Cyclotella delicatula*, del tipo funcional (codón)* ***B****, dominando la comunidad en toda la columna óxica de agua durante el invierno. Esta especie persiste en primavera, pero ligada entonces a capas más profundas. En primavera, al principio de la estratificación térmica, se produce el desarrollo en las capas superiores de picocianobacterias, criptofíceas, y dinoflagelados, correspondientes a los grupos funcionales* ***Z****,* ***Y****, y* ***Lo****, quedando las dos primeras progresivamente recluidas en la oxiclina a medida que la estratificación avanza. Con el avance del periodo de estratificación se desarrollan densas poblaciones de clorofíceas de los grupos funcionales* ***F****,* ***X****, y* ***J*** *en el epilimnion y el metalimnion, mientras que hacia final del verano se seleccionan especies de* Cosmarium*, pertenecientes al grupo funcional* $\mathbf{N_A}$*, que pasan a ser dominantes hasta que la mezcla otoñal vuelve a dar paso a las diatomeas. En su conjunto, la fuerte estratificación de la columna de agua permite crear diferentes nichos con una distribución vertical diferencial del fitoplancton durante los periodos de estratificación, lo que favorece su diversidad en el conjunto de la columna de agua. Sin embargo, diversos tipos de perturba-*

ciones, tales como episodios de fuertes lluvias, movimientos horizontales de masas de agua y, en especial, el fenómeno de blanqueamiento que ocurre todos los años hacia mitad del verano, alteran en cierta medida el patrón sucesional.

Palabras clave: *Fitoplancton, sucesión, grupos funcionales, estratificación, lagos meromícticos*

INTRODUCTION

The seasonal composition of the phytoplankton assemblages follows regular patterns within the annual cycle (Padisák *et al.*, 1998; Morabito *et al.*, 2003; Salmaso & Padisák, 2007). Relatively few species are dominant at any given time, and seasonal species substitution occurs according to the contrasting strategies adopted by the different species (r-K Margalef, 1979, Sommer, 1986; R-C-S Reynolds, 1997) The process is mainly governed by changes in light and nutrient availability (Margalef, 1979; Reynolds, 1984) in a recurrent annual sequence. Reynolds *et al.* (2002), and many other authors thereafter (e.g. Kruk *et al.*, 2002; Morabito *et al.*, 2003; Huszar *et al.*, 2003; Padisák *et al.*, 2009; Salmaso *et al.*, 2015), have developed a phytoplankton classification into functional groups, whose associations are mainly determined by environmental conditions such as the physical properties of the water column and the trophic status. This functional classification of freshwater phytoplankton is based on their preferences on features related to seasonal stratification and mixing changes, but adds the trophic characteristics of the system and its different strata, as well as the vertical phytoplankton distribution.

After studying the phytoplankton abundance and biomass dynamics, the present work analyses the characteristic phytoplankton succession at the Laguna de La Cruz, a meromictic lake in Cuenca (Central-Eastern Spain), both at taxonomic and functional levels. Special attention is paid to the time-course of diversity as a descriptive parameter for the progression of succession. The establishment of both temporary and permanent pycnoclines in this lake is of paramount importance as this creates a marked stratification of the water column, with an anoxic monimolimnion present all year long, and a microaerobic or anoxic hypolimnion developing over the monimolimnion during the stratification period. Our study analyses the differences in phytoplankton succession in the different strata (mixolimnion during mixis, and epilimnion, metalimnion and oxycline during stratification). In order to better visualise the main pattern of seasonal succession and to clarify the factors determining the spatio-temporal distribution of phytoplankton assemblages in this lake, several multivariate statistical analyses were carried out, aimed at describing the dynamic patterns of phytoplankton.

This was the last manuscript Prof. Maria Rosa Miracle was working on before she passed away. Here, she was making an effort to synthesise long-term observations that our group made in Laguna de La Cruz, one of her best studied lakes. With this manuscript her collaborators have tried to conclude her task, aiming to find the ultimate pattern of phytoplankton succession in one of her preferred study lakes.

MATERIAL AND METHODS

Study area

Laguna de La Cruz (Lake La Cruz) is a meromictic sink hole lake located in the south of the “Serranía de Cuenca”, at an altitude of 1032 m a.s.l. Several holomictic lakes are also found in the sink holes (“torcas”) within this area, known as the “Torcas de Cañada del Hoyo” (UTM30SWK9627) (Roca *et al.*, 2000; Rodrigo *et al.*, 2000, 2001). Laguna de La Cruz occupies the lower half of the frustoconical sink hole. During the study period, its surface covered around 1 Ha, with a maximum depth between 21 m and 22 m. This lake is mostly fed by groundwater, and presents a monimolimnion rich in calcium bicarbonate, delimited by a permanent chemocline located between 16 m and 18 m of depth, that is defined by a conductivity gradient. In addition to the chemical stratification, the lake presents a marked thermal stratification beginning by middle spring, with a thermocline deepening as season advances. This process allows the forma-

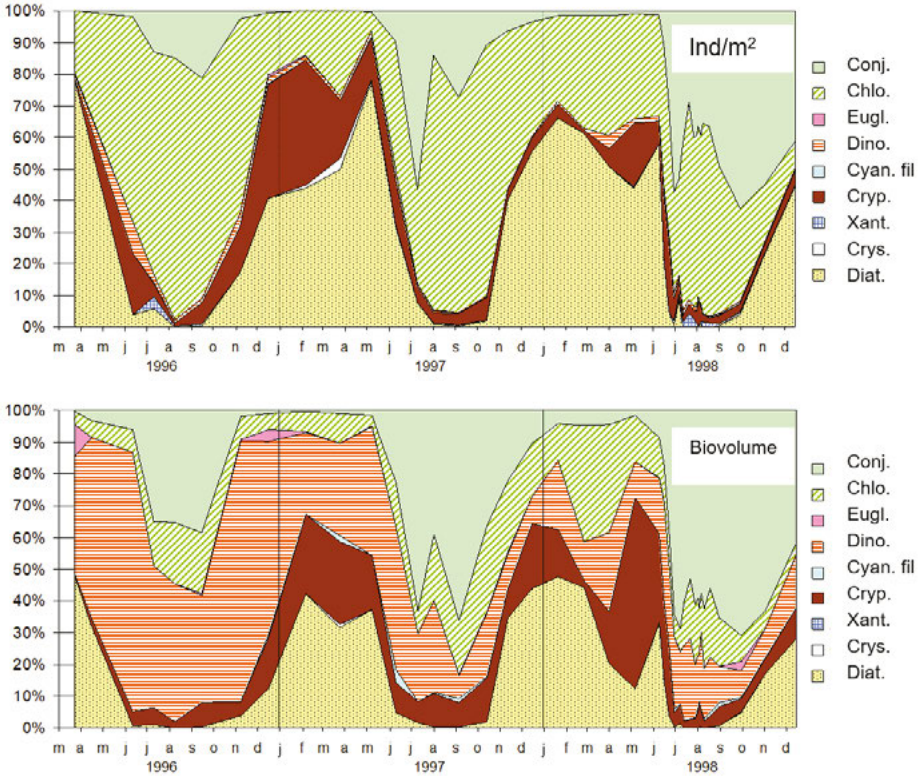


Figure 1. Relative contribution (%) of the different nano- and micro-phytoplankton groups integrated for the whole oxic water column of Laguna de La Cruz throughout the study period. The results are given as a percentage (%) of the individuals/m^2 (above) and of biovolume (below). *Contribución relativa (%) de los diferentes grupos de nano- y microfitoplancton integrados para toda la columna de agua óxica de la Laguna de La Cruz a lo largo del período de estudio. Los resultados se proporcionan como porcentaje (%) de los individuos/m^2 (arriba) y como biovolumen (abajo).*

tion of an anoxic hypolimnion which, in turn, increases in volume to reach the lower limit of the metalimnion in summer months. In summer, the oxic-anoxic interphase is located at around 11 m (Camacho *et al.*, 2017). Limnological information about the characteristics of this lake dates back to studies performed from 1980s onward (e.g. Vicente & Miracle, 1988; Miracle *et al.*, 1992; Rodrigo *et al.*, 2001). However, a broader and detailed survey of the temporal and spatial patterns of temperature, light penetration, chlorophyll-*a* concentration, and picocyanobacerial abundance, was performed monthly from 1996 to 1998 (Camacho *et al.*, 2003a). Conversely, however, almost no data on nano- and microphytoplankton have been available for these years, though hundreds of samples were collected. We here cover this gap, by analysing these samples, in order to unveal the community patterns of phytoplankton in Lake La Cruz. This is among the soundest studies so far performed on the phytoplankton community of a meromictic lake (Zadereev *et al.*, 2017).

Sampling and analytical and statistical methods

Sampling was done monthly at the lake's central point, beginning in March 1996 and ending in December 1998. In addition to monthly sampling, additional samples were collected with a weekly frequency in the summer 1998, to better investigate the usual whitening event, caused by tumultuous calcium carbonate precipitation, repeatedly occurring every summer (Camacho *et al.*, 2017).

The samples used to study phytoplankton were collected with a double cone fine-layer sampler (Miracle *et al.*, 1992) or a Ruttner bottle depending on the stratification status, then pooled into glass topaz 250 ml bottles and immediately fixed with Lugol (Wetzel & Likens, 2000). Different depths of the vertical profile covering the different strata were sampled. At each sampling event the number of samples varied according to strata thickness: 2-3 samples from the epilimnion, 3-7 samples from the metalimnion, 1-4 from the oxic hypolimnion and 1 sample from the oxic-anoxic interphase. In order to determine the sample collection depths, the corresponding vertical profiles of water electrical conductivity and temperature (WTW LF-191 conductivity meter with a temperature probe), dissolved oxygen (WTW digital meter, model OXI-91), light penetration (Li-Cor model Li-1000), and pH and Eh (with two electrodes ORION model 81-02SC and ORION model 96-78, respectively), were obtained *in situ* at the sampling point. Simultaneously, samples were collected to analyse chlorophyll-*a*, nutrients and dissolved salts concentrations. All these data, as well as the results of the picocyanobacterial counts, can be found in Camacho *et al.*, 2003a.

For phytoplankton quantification, the Utermöhl sedimentation method (1958) was followed by subsequent counting with a Zeiss inverted microscope (CARL ZEISS, Germany 473012-9902). Phytoplankton species were identified mainly following Huber-Pestalozzi (1968), Förster (1982), Komárek and Fott (1983), Krammer and Large-Bertalot (1986-1988-1991a-1991b) and Popovský and Pfiester (1990). In order to calculate algal biovolumes, the formulae of the volumes of the nearest geometric forms, or their corresponding combinations, were applied according to Rott (1981) and Hillebrand (1999). Phytoplankton species vere assigned to different functional groups following Reynolds *et al.* (2002) and Padisák *et al.* (2009). The point samples data were integrated per square meter of the oxic water column as follows:

$$T_i = \sum_{k=1}^{n} X_{ik} \left(\frac{Z_{k+1} - Z_{k-1}}{2} \right)$$

where:

T_i = total individuals of the species i in a water column of one square metre section, ranging from the surface to the oxygen extinction depth.

k = each sampling depth

Z_{k+1} = next depth below depth k

Z_{k-1} = previous depth above depth k.

X_{ik} = number of individuals per cubic metre of species i that was measured at depth Z_k.

Diversity was computed using the Shannon diversity index (Shannon & Weaver, 1963; Margalef, 1979) for each individual sample depth as well as for the phytoplankton abundances of all species integrated within the oxic water column. The heterogeneity of the phytoplankton community within the water column (Margalef, 1979), D_β, was expressed as the ratio between the effective number of species, i.e., exp (H') (Jost, 2006), which resulted from the diversity data of the integrated water column and the mean of those corresponding to the different sampling depths. H' is the Shannon-Wiener entropy, as follows:

$$H' = -\sum_{i=1}^{S} p_i \ln p_i \text{(Shannon \& Weaver, 1963).}$$

Statistical analyses were run using SPSS for Windows v11 and MVSP v3 software (Multivariate Statistical Package for Windows, Kovach, 1998). A Principal Component Analysis (PCA) was performed for the primary ordination of phytoplankton assemblages to unveal the successional pattern. Thereafter, a Canonical Correpondence Analysis (CCA) was performed to relate the main environmental explanatory variables with the phytoplankton assemblages. Normalisation (log x+1) was performed in both cases when convenient after checking data distribution.

RESULTS

Seasonal variation of the different algal groups

When considered in terms of algal abundance, a clear alternation occurred during the annual cycle between diatoms and chlorophytes. Diatoms were the most abundant phytoplankton group during the mixing periods, whereas chlorophytes were the most abundant during summer stratification

(Fig. 1). Diatoms presented their highest proportions in December, January and February, although they were also dominant in spring in years 1997 and 1998, when heavy spring rainfall caused lower stability of the water column. Additionally, diatoms growth at the bottom of the metalimnion towards the end of spring also contributed to these spring dominances. Conversely, chlorophytes dominated in summer, with their maxima always occurring in August (83 %, 81 % and 54 % of phytoplankton cells, respectively, in the three consecutive years). The relative contribution of conjugated algae (Zygnematophyceae) began to increase after chlorophytes developed, with maxima at the end of summer. Dinophytes were particularly important when biomass (biovolume) was considered; they reached higher proportions towards the end of winter and in early spring, coinciding with the beginning of thermal stratification (Fig. 1). Contrastingly, cryptophytes reached their highest proportions in spring and summer, though they were mostly distributed close to the oxycline. Among the groups with much lower relative contribution, chrysophytes and xantophytes were also detected. Though modestly, chrysophytes grew at the beginning of either the spring or the autumn, whereas xantophytes reached their highest proportions by mid-summer after the drop of diatoms in spring, but disappeared during the

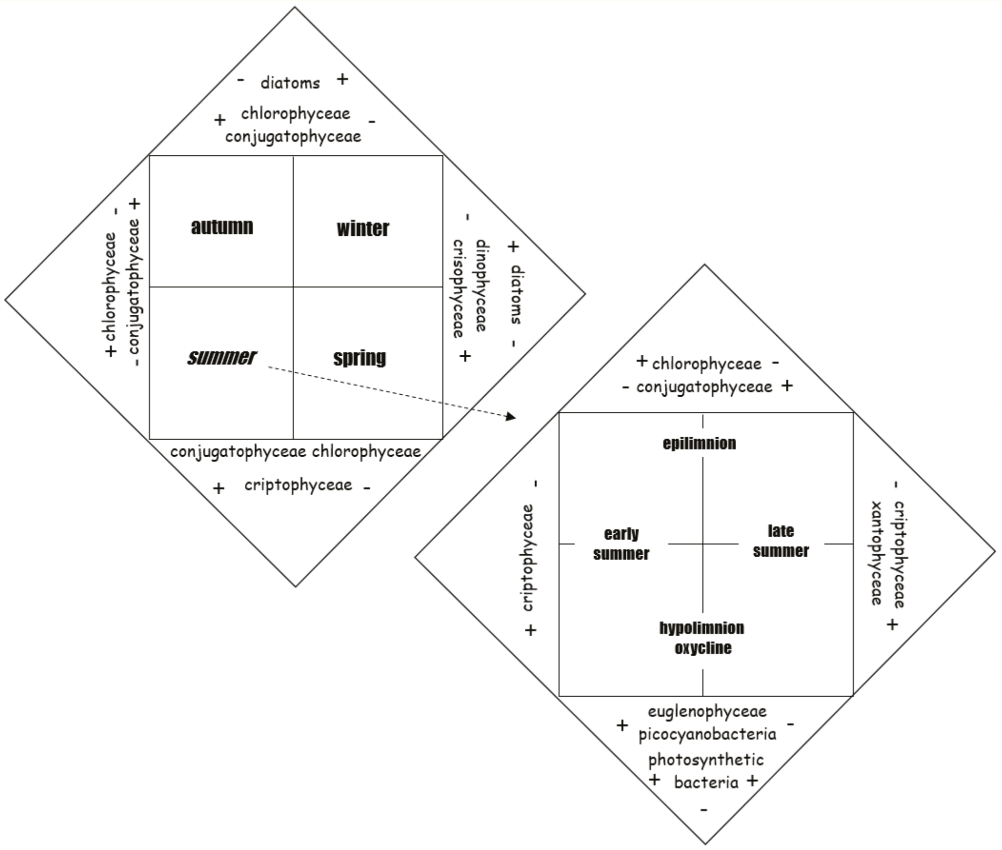


Figure 2. The "Mandala" (Margalef, 1979) that represent the seasonal succession of the phytoplankton community in Laguna de La Cruz. Squares represent the seasons when the main groups mentioned in each square side are present, along with their dynamics using the + and - signs. A secondary "Mandala" is shown on the right only for summer, with the vertical distribution added. *"Mandala" (Margalef, 1979) que representa la sucesión estacional de la comunidad de fitoplancton en la Laguna de La Cruz. Los cuadrados representan las estaciones en las que están presentes los grupos principales mencionados en cada lado del cuadrado, junto con su dinámica mediante los signos + y -. A la derecha se muestra un "Mandala" secundario solo para el verano, con la distribución vertical agregada.*

mixing period. Euglenophytes, appearing mainly in autumn, and filamentous Cyanobacteria, ocasionally present in summer, were also very scarce, and their modest appearance always occurred close to the oxyline. Contrastingly, picocyanobacteria (*Synechococcus*) accounted for an important part of the primary producers, though they are not detailed in this paper since their dynamics was previously described by Camacho *et al.* (2003a).

Succession of the most important phytoplankton taxa

Succession in the phytoplankton of Laguna de La Cruz started with autumnal mixing, with the development of an almost mono-specific *Cyclotella delicatula* population, corresponding to the codon **B**, accounting for nearly 90 % of diatom's biovolume. In the first part of the mixing period, in autumn, some pennated diatoms from genus *Fragilaria* (codon **P**), as well as cryptophytes like *Rhodomonas minuta*, *Cryptomomas phaseolus* and *C. obovate* (codon **Y**), also appeared. Species that develop at the end of summer, but also grow in autumn, were characteristic, such as *Cosmarium bioculatum* (codon $\mathbf{N_A}$), *Pseudoquadrigula* sp. (codon **F**), *Lagerheimia* sp. (codon **J**) and *Peridinium umbonatum* (codon **Lo**).

Cyclotella delicatula also dominated winter phytoplankton assemblages, co-occurring with *Gymnodinium* cf. *bogoriense*. Fast growth of *Synechococcus* sp. (Picocyanobacteria. belonging to codon **Z**) occurred in late winter and early spring through the oxic water column, though they were restricted to the metalimnion in summer. Some species of *Rhodomonas* (*R. minuta*, *R. lacustris*) belonging to codon **X2**, and of *Cryptomonas* (*C. obovata*, *C. phaseolus*; codon **Y**) also developed in late winter, but they were later restricted to the oxycline, where they reached their maximum abundance during stratification. With the advent of the stratification, chlorophytes became dominant, especially *Kirchneriella irregularis*, *Pedinomonas minor*, *Scenedesmus praetervisus*, *Lagerheimia quadriseta*, *Chlorella vulgaris*, *Tetraedron minimum* and *Pseudoquadrigula* sp. Through summer, larger sized, S-strategists, algae became more abundant, such as *Ceratium hirundinella*, *Peridinium cinctum*, and species of genus *Cosmarium* (e.g., *C. abbreviatum*, *C. laeve*, *C. meneghinii* and *C. bioculatum*). Following Reynolds' functional classification, the species of *Peridinium*, mainly *P. cinctum*, belong to the codon **Lo**, which dominates in the epi-metalimnion of the stratified water columns along with *C. hirundinella*. The species of genus *Cosmarium* belong to group $\mathbf{N_A}$, where they are usually found in association with pennate diatoms (e.g. *Tabellaria* spp.), though these pennate diatoms (in our case *Fragilaria* and *Nitzschia*) only grew significantly in the Laguna

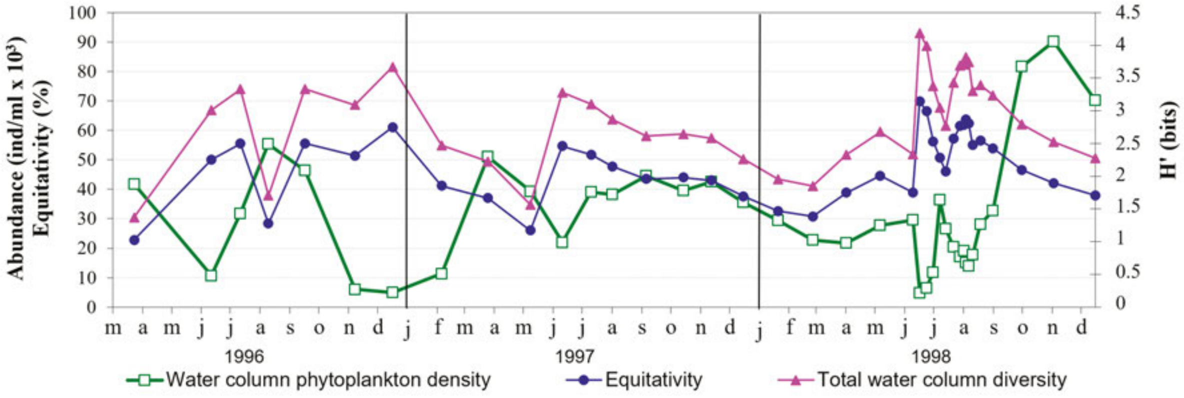


Figure 3. Phytoplankton abundance (ind/ml x 10^3) in the water column of Laguna de La Cruz, Shannon diversity index (bits) and evenness (equitativity, %) of the whole water column. *Abundancia de fitoplancton (ind/ml x 10^3) en la columna de agua de la Laguna de La Cruz, índice de diversidad de Shannon (bits) y equitatividad (%) en toda la columna de agua.*

de La Cruz in autumn of 1998, when the *Cosmarium bioculatum* population reached its highest values. Following the functional classification of phytoplankton by Reynolds *et al.* (2002), the succesional sequence in the epi-metalimnion is:

B, Z → (X, F, J,) → Lo → N_A → B, Z

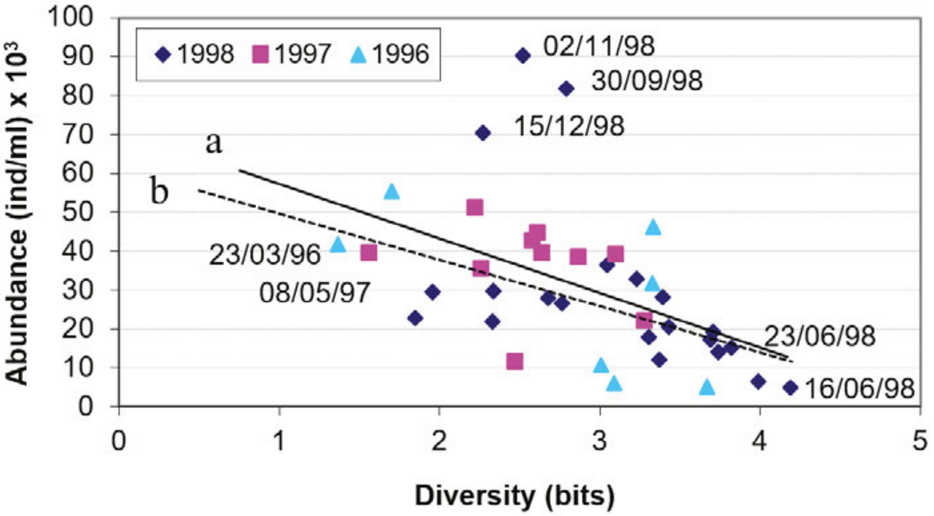
In our work, phytoplankton succession in Laguna de La Cruz was differentially considered in two different strata, the epi-metalimnion and the oxycline. Succession in the oxycline advances much slower, and species of coda **Y**, **Z** and **W1**, which correspond to Cryptophyta, picocyanobacteria and Euglenophyta respectively, replace those of groups **B** and **Z** during the stratification period. The sequence at the oxycline would be as follows:

B, Z → (Z, X2, → Y →W1) → B, Z

Figure 2 shows the Mandala representing phytoplankton succession in Laguna de La Cruz during the 1996-1998 period, along with a second Mandala representing the characteristic vertical distribution of phytoplankton during the stratification period. These mandalas show the pattern of diatoms maxima occurring in winter, that of chlorophytes in summer, conjugated algae at the end of summer and autumn, and cryptophytes in summer, though the later concentrated at the oxycline.

Seasonal variation of phytoplankton abundance, biomass and diversity

Our study clearly shows an inverse relationship between phytoplankton abundance and diversity (Figs. 3 and 4). This relationship is statistically significant, $r^2 = -0.26$, $p < 0.01$, (Fig. 4), even strongest ($r^2 = -0.506$, $p < 0.01$) when the data from autumn 1998, with moderate diversities but very high abundance of *Cosmarium bioculatum* and *Cyclotella delicatula*, were excluded from the correlation. At the beginning of spring, abundance increases and phytoplankton diversity



a: n=38, r= -0.611, p<0.01, r^2 =0.26

b: without autumn 1998, n=35, r= -0.506, p<0.01, r^2 =0.37

Figure 4. (a) Relationship between the phytoplankton abundance (ind/ml x 10^3) and the Shannon diversity index (bits) of phytoplankton for the whole water column of Laguna de La Cruz for all samples of the study period, or (b) excluding the samples of autumn 1998. *(a) Relación entre la abundancia de fitoplancton (ind/ml x 10^3) y el índice de diversidad de Shannon (bits) del fitoplancton para toda la columna de agua de Laguna de La Cruz para todas las muestras del período de estudio, o (b) excluyendo muestras de otoño de 1998.*

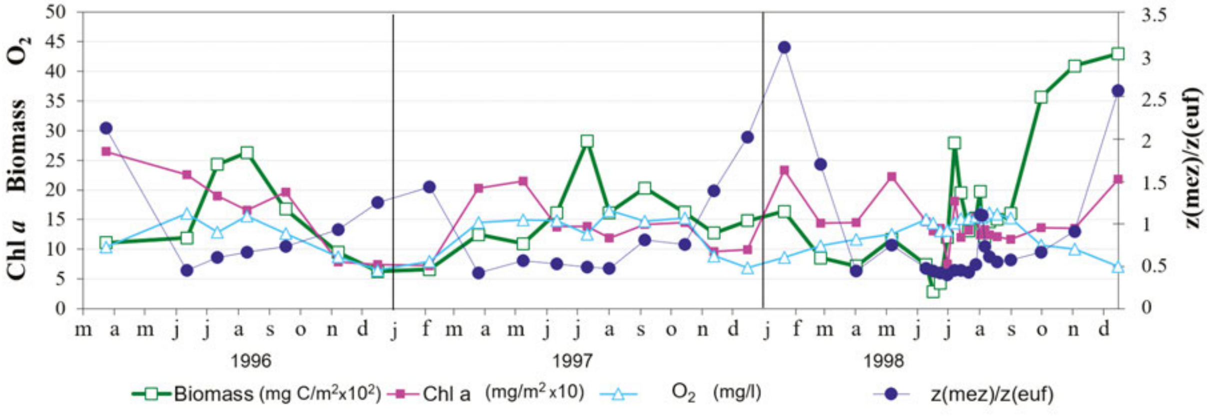


Figure 5. Integrated water column phytoplankton biomass (mg C/m^2 x 10^2), integrated chlorophyll-*a* concentration (mg/m^2 x 10), maximum concentration of dissolved oxygen (mg/l), and the quotient between mixing depth and the depth of the euphotic zone, during the studied period in Laguna de La Cruz. *Biomasa fitoplanctónica integrada de la columna de agua (mg C/m^2 x 10^2), concentracion de clorofila-*a *integrada (mg/m^2 x 10), concentración máxima de oxígeno disuelto (mg/l), y cociente entre la profundidad de mezcla y la profundidad de la zona eufótica, durante el período estudiado, en la Laguna de La Cruz.*

drops. Yet, towards the end of spring and the beginning of summer, a sudden change occurs and the opposite situation prevails for a short period, with a drop in abundance and an increase in diversity. Conversely, halfway through summer, nutrient inputs occurred due to the horizontal transport from the shores, resulting in growth peaks accompanied by reduced diversity, although diversity decreases throughout autumn. Chlorophyll-*a* concentrations do not always match phytoplankton biomass (Fig. 5), being quite dependent on the size and relative chlorophyll content of the dominant taxa at each period, as well as on the relative contribution to the chlorophyll-*a* pool of picocyanobacteria (see Camacho *et al.*, 2003a for detailed data on picocyanobacteria abundance).

The seasonal development of phytoplankton in Laguna de La Cruz varied, being influenced by the weather conditions, in different years. Along the studied years, maximum phytoplankton abundance differed according to the intensity and depth of vertical mixing, represented by the ratio between the depth of the mixing zone (Z_{mez}) and the euphotic depth (Z_{euf}). Indeed, this ratio for the 1997-1998 mixing period (2.05) doubled that of the previous (1996-1997) hydrological cycle (1.25) (Fig. 5). The stable weather conditions in the autumn of 1998 favoured a greater stability and coincided with higher biomass in autumn compared to the previous years, though diversity still remained high in this period (Figs. 3 and 4). During the mixing periods, especially in winter and the beginning of spring, phytoplankton diversity was lower than the average of the stratification periods (Fig. 3). Furthermore, the lowest phytoplankton diversity within any water column layer during stratification was found in the epilimnion, in such a way that the aforementioned processes, i.e., variations in diversity through sporadic nutrient inputs, were much more visible in this layer. Constrastingly, the oxic meta-hypolimnion generally presented higher diversity than the epilimnion during the well-established stratification in summer (Fig. 6), when many phytoplankton species developed at these relatively stable layers that show differential conditions at different depths, yet differentially offering contrasting niches with respect to light and nutrient availablility. Nevertheless, the oxic-anoxic interphase (oxycline) also presented high values of the Shannon diversity index, partly supported by the sedimentation of several species that accumulate in this area by equalizing buoyancies to the water density gradient (see Camacho, 2006, for details).

A proxy for β-diversity, the heterogeneity of the phytoplankton community within the water column (D_β), was related to the difference between the surface temperature and that of the bottom of the lake (Fig. 7 - the arrows show the seasonal pattern displayed by this relationship). All sampling events corresponding to the lake's mixing period remain in the lower left quadrant, wtih low temperatures linked to low heterogeneity. Autumn samplings can be divided into two groups: those before mixing (top left part of the chart) showing higher D_β, and those after mixing (lower left part of the chart) with lower D_β. Spring and summer samplings, with large vertical differences in temperature, remain on the right-hand side of the figure, with D_β values above 1, except for the sampling dates that coincided with algal blooms. These blooms were caused either by the development of: i) *Lagerheimia quadriseta* in the epilimnion and *Kirchneriella irregularis* in the hypolimnion in August 1996, and to ii) the development of populations of *Cyclotella delicatula*, *Cyclotella distinguenda* and *Pedinomonas minor* at the beginning of June

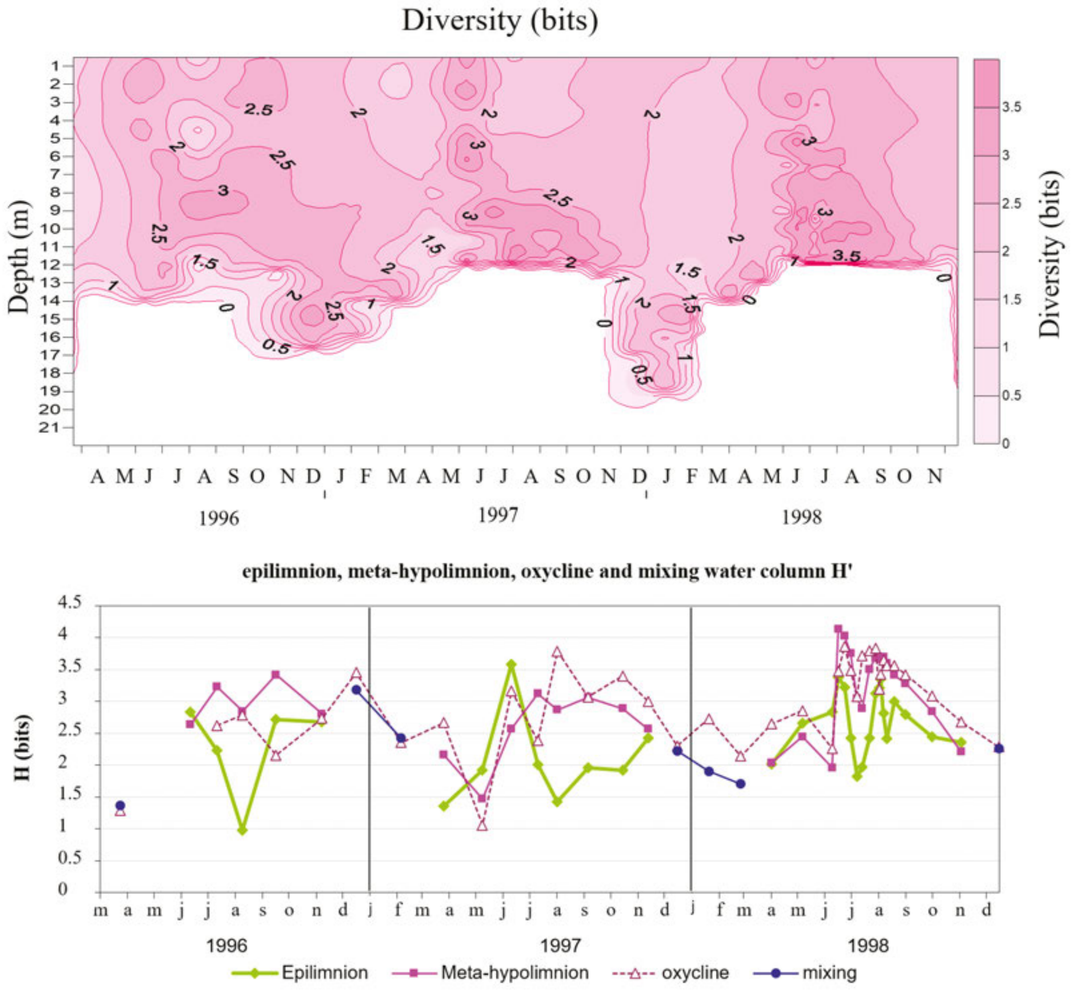


Figure 6. (Above) Isopleths showing the spatio-temporal variation of the Shannon diversity index (bits) in Laguna de La Cruz throughout the study period; and (Below) the Shannon diversity index (bits) calculated for the samples of the different layers: epilimnion, meta-hypolimnion, oxycline, and mixolimnion up to the oxycline during the mixing period. *(Arriba) Isolíneas que muestran la variación espacio-temporal del índice de diversidad de Shannon (bits) en la Laguna de La Cruz durante todo el período de estudio; y (abajo) índice de diversidad de Shannon (bits) calculado para las muestras de las diferentes capas: epilimnion, meta-hipolimnion, oxiclina, y mixolimnion hasta la oxiclina durante el período de mezcla.*

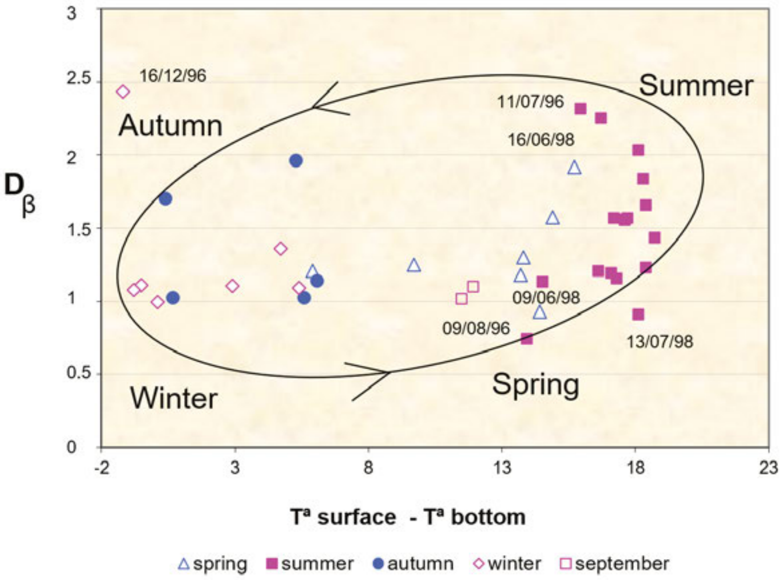


Figure 7. Relationship between the heterogeneity of the phytoplankton assemblages - D_β - (total column H' exponential / sample mean H' exponential), and the difference between the surface temperature and the bottom lake temperature. *Relación entre la heterogeneidad de los ensamblados de fitoplancton - D_β - (toda la columna H 'exponencial/ muestra media H' exponencial), y la diferencia entre la temperatura de la superficie y la del fondo del lago.*

1998, and to the the massive development of *Chlorella vulgaris* and *Cosmarium abbreviatum* in mid-July 1998. After the whitening event commonly occurring in August (Camacho *et al.*, 2017), the phytoplankton assemblages altered its vertical structure, being more homogeneously distributed through the water column.

Statistical analyses

In order to globally study the behaviour of the phytoplankton assemblages during the annual cycle, a principal component analysis (PCA) was performed (Fig. 8) using the abundance values (log-transformed) of the 31 most frequent phytoplankton species appearing in 1998, the sampling year that included more details (209 cases -depths- distributed within 21 sampling events). Additionally, a canonical correspondence analysis (CCA) was also done with the same data, where environmental variables were additionally considered in order to unveal the relationship between the phytoplankton assemblages and key environmental variables (Fig. 9).

The first axis resulting from the PCA explained 25 % of variance, whereas the second axis explained an additional 15 %. Samples distributed in the space defined by these two axes according to a sequence which followed the seasonal succession, (horizontal axis separated spring and autumn samples) (Fig. 8). In the ordination sequence, the summer samples corresponding to the oxycline followed those of spring. This shows the fact that some species thriving in upper layers in spring were confined to the oxycline upon stratification later on. This succesional sequence also shows the summer epilimnetic samples as the closests to the autumn samples on the other side of horizontal axis, which indicates that the characteristic species of upper layers in summer remain in autumn. Within the sequence, autumn samples are followed by the samples of the mixing period, which again connect with the spring samples, showing a nice drawing of the seasonal succession of phytoplankton in Laguna de La Cruz. The vertical axis also clearly separates the samples from the mixing period, on the upper part of the chart, from those of stratification, mainly found in the lower part.

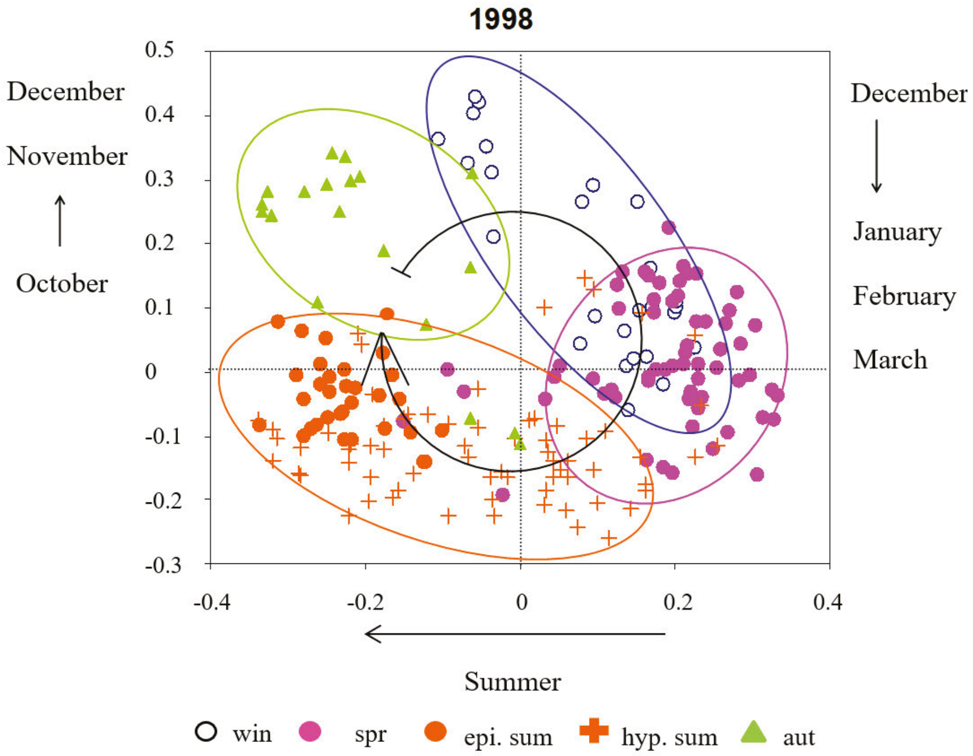


Figure 8. Situation of the phytoplankton samples taken in 1998 in Laguna de La Cruz in the space defined by the first two factors of the PCA, showing differentially the winter, spring, summer epilimnion, summer hypolimnion, and autumn samples. *Situación de las muestras de fitoplancton tomadas en 1998 en la Laguna de La Cruz en el espacio definido por los dos primeros factores de la PCA, que muestra diferencialmente las muestras de invierno, primavera, epilimnion de verano, hipolimnion de verano y otoño.*

The CCA (Fig. 9) sequentially ordered both the species (Fig. 9a) and the samples (Fig. 9b) by grouping those from winter (December, January, February and March), from early-middle spring (April, May and the first week of June), from late spring (weeks 2 and 3 of June), from summer (last week of June, and July and August) and from autumn (October and November). The CCA also separated samples from upper depths from those of the oxycline, and also counterposed samples from the mixing period to those of stratification.

From the CCA using the species data (Fig. 9a), the following phytoplankton assemblages were unveiled: 1) the characteristic species of the mixing period, *Cyclotella delicatula*, *C. distinguenda*, *Gymnodinium bogoriense* and *Kirchneriella irregularis*, which are distributed homogeneously in the water column, but are not characteristic of the oxic-anoxic interphase, and are related mainly with nitrate, which was more abundant during the mixing period (see Camacho *et al.*, 2003a for detailed data on water chemistry); 2) *Cryptomonas erosa* and *Scenedesmus praetervisus*, whose densities were higher in spring, 3) *Scenedesmus ecornis*, *Oocystis lacustris*, *Cryptomonas* sp., *Cryptomonas obovata* and *Rhodomonas lacustris*, which, despite of being present throughout the mixing period throughout the whole water column, were relegated to the metalimnion with the advent of stratification; 4) *Rhodomonas minuta*, *Cryptomonas phaseolus* and *Ankistrodesmus gelifactum*, which are characteristic species of the oxycline, mostly developing during the stratification period. *Euglena acus* remains away as it just appeared in very few oxycline samples, which coincided with the maxima of picocyanobacteria found at these depths. On the other hand, some species arise as characteristic of the stratificacion period, though no clear preference was observed by these species for a given layer when compared between the upper layers and the oxycline. These species are *Cosmarium laeve*, *C. bioculatum*, *C. meneghinii*, *Pseudoquadrigula* sp. and *Trachydiscus minutus*. Comparatively, *Chlorella vulgaris* and *Lagerheimia ciliata* appear as typical epilimnetic or metalimnetic species, respectively, whereas *Peridinium umbonatum* and *Pedinomonas minor*, however, are typical epilimnetic species, with maximum abundance in spring. *Monoraphidium subclavatum* and *Cosmarium abbreviatum* peaked both at the beginning of the summer and, later on, in autumn. Comparatively, the most ubiquitous species remain in a central position of the CCA chart (Fig. 9a).

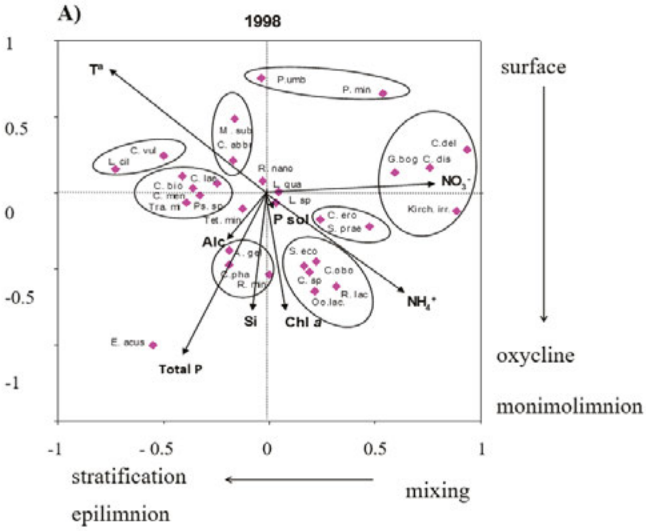
The groups of the species resulting from both the PCA and CCA describe the succession of the most relevant phytoplankton species in Laguna de La Cruz, and coincide with the functional groups previously described.

DISCUSSION

The seasonal succession of phytoplankton in Laguna de La Cruz only partly resembles that of other stratified temperate lakes (Margalef, 1979; Reynolds, 1997). As previously reported (Kiss *et al.*, 2007), phytoplankton succession in Laguna de La Cruz begins with diatoms becoming dominant during the autumn mixing period, especially *C. delicatula*. After a maximum in spring, diatoms virtually disappear, while other species replace them. Phytoplankton succession has been investigated in many lakes such as e.g., in the deep, oligotrophic and alkaline Lake Stechlin (Padisák *et al.*, 1998), where a similar pattern was reported. However, the meromictic nature of the Laguna de La Cruz, with its partial vertical mixing above the monimolimnion by the establishment of an overlying oxycline during the stratification period, allows for the occurrence of a deep stratum where diatoms remain until mid-spring, thus the succession pattern differs from other deep lakes in the vertical distribution of dominant populations. As a general framework, it is noteworthy that the density and viscosity of the water governed by temperature variation, may strongly determine the seasonal sinking behaviour of phytoplankton and determine its seasonal succession. Additionally, changes in cell or colony size also could enable some species to overcome temperature-dependent changes in water density and viscosity and adjust their sinking velocities in the different seasons (Zohary *et al.*, 2017).

The dominance of diatoms during the mixing period, when nutrients are renewed, is associated with the characteristics of many species of the genus *Cyclotella* as r-strategists that follow general phytoplankton succession patterns (Margalef, 1979; Sommer, 1986), and belong to in the C-category defined by Reynolds (1997). Following the functional phytoplankton classification, small centric diatoms, like *C. delicatula*, would belong to the codon **B**, typical of vertically mixed, mesotrophic small-medium size lakes (Padisák *et al.*, 2009), that includes species sensitive to silica deficiency and to stratification, yet tolerant to low light intensity. Hence, they can remain in the metalimnion for some time when stratification begins (Reynolds *et al.*, 2002).

The CCA confirms that the *Cyclotella* dynamics in Laguna de La Cruz can be associated with variations in the concentration of silica, as it has been well established by other authors (e.g. Flynn & Martin-Jézéquel, 2000; Cetin & Sen, 2004) that also observed silica depletion following diatoms' peaks. Another factor that favours diatom development is turbulence, which allows them to remain in the photic zone. When turbulence decreases, as it occurs in Laguna de La Cruz with the onset of the thermal stratification, diatoms start to be replaced by other algae. Picocyanobacteria (Camacho *et al.*, 2003a), as well as *Rhodomonas* and small chlorophyceae, peak later than diatoms, in spring. The faster growth of these picocyanobacteria, *Synechococcus*, occurred in late winter and early spring through the oxic water column, though they were restricted to the metalimnion in summer (Camacho *et al.*, 2003a). Picocyanobacteria (codon **Z**), *Rhodomonas* (codon **X2**), as well as *Cryptomonas* (codon **Y**), all of them tolerating low light availability (Gasol *et al.*, 1992, 1993; Camacho *et al.*, 2001, 2003a), dominated the lower metalimnion and the oxycline during stratification. Nutrient exhaus-



A. gel: *Ankistrodesmus* cf. *gelifactum*; C. abbr: *Cosmarium abbreviatum*; C. bio: *Cosmarium bioculatum*; C. dis: *Cyclotella distinguenda*; C. er: *Cryptomonas erosa*; C. lae: *Cosmarium leave*; C. men: *Cosmarium meneghinii*; C. sp: *Cryptomonas* sp.; C. vul: *Chlorella vulgaris*; C. del: *Cyclotella delicatula*; C. obo: *Cryptomonas obovata*; C. pha: *Cryptomonas phaseolus*; E. acus: *Euglena acus*; G. bog: *Gymnodinium* cf. *bogoriense*; Kirch. irr: *Kirchneriella irregularis*; L. cil: *Lagerheimia ciliata*; L. qua: *Lagerheimia quadriseta*; L. sp: *Lagerheimia* sp.; M. sub: *Monorraphidium subclavatum*; Oo. Lac: *Oocystis lacustris*; P. min: *Pedinomonas minor*; P. umb: *Peridinium umbonatum*; Ps. sp: *Pseudoquadrigula* sp.; R. lac: *Rhodomonas lacustris*

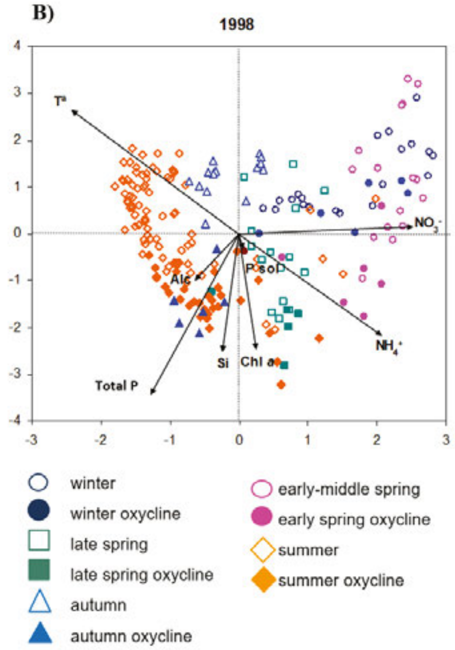


Figure 9. a) Situation of the main phytoplankton species and vectors that correspond to the different environmental variables (ammonium, nitrate, soluble phosphorus, silicate, temperature, total phosphorus and chlorophyll-*a*), from samples of 1998, in the space defined by the first two factors resulting from the CCA. b) Situation of the samples and vectors that corresponded to the different environmental variables from samples of 1998 in the space defined by the first two factors that resulted from the CCA. *a) Situación de las principales especies de fitoplancton y vectores que corresponden a las diferentes variables ambientales (amonio, nitrato, fósforo soluble, silicato, temperatura, fósforo total y clorofila-*a*), a partir de las muestras de 1998, en el espacio definido por los dos primeros factores resultantes de la CCA. b) Situación de las muestras y vectores que correspondieron a las diferentes variables ambientales de las muestras de 1998 en el espacio definido por los dos primeros factores que se obtuvieron de la CCA.*

tion in upper layers (Camacho *et al.*, 2003a) poses nitrogen limitation to picocyanobacteria (Camacho *et al.*, 2003b), while the drop in both the *Rhodomonas* and Chlorophyceae populations towards the end of spring could also be favoured by the grazing by the cladoceran *Diaphanosoma brachyura*, whose population considerably increases at this time (Boronat, 2003). Both zooplankton grazing and nutrient exhaustion in the epilimnion would explain the phytoplankton decrease at the end of spring, as predicted by the PEG model (Sommer *et al.*, 1986).

During the summer stratification period, small chlorophytes and species of the genus *Cosmarium* account for phytoplankton recovery in the epilimnion, With the advent of the stratification these chlorophytes became dominant, with species like *Pedinomonas minor*, *Lagerheimia quadriseta*, *Kirchneriella irregularis*, *Tetraedron minimum*, *Scenedesmus praetervisus*, *Chlorella vulgaris*, and *Pseudoquadrigula* sp. These are C-strategist according to Reynolds' (1997) classification of phytoplankton life-strategy, showing a good nutrient assimilation capacity, and a high replication ability after abrupt changes in the water column conditions. They included species belonging to the codon **F**, which groups algae tolerating low nutrient concentrations and high turbidity; to codon **X**, whose species tolerate stratification but are sensitive to nutrient shortage and feeding by zooplankton (*Chlorella*, *Tetraedron*); and the **J** group, which includes Scenedesmus. Throughout summer, when nutrients become increasingly scarce in surface layers and the zooplankton population increases (Boronat, 2003), larger sized algae, S-strategists, were selected; e.g., *Ceratium hirundinella*, *Peridinium cinctum* and species of the genus *Cosmarium*, (basically *C. abbreviatum*, *C. laeve*, *C. meneghinii* and *C. bioculatum*). Following Reynolds' functional classification, the species of genus *Peridinium*, mainly *P. cinctum*, belong to the codon **Lo**, which dominates in the epi-metalimnion of the stratified water columns along with *C. hirundinella*. The species of genus *Cosmarium* belong to group $\mathbf{N_A}$, and are usually found in association with pennate diatoms (e.g. *Tabellaria* spp.), though these pennate diatoms (in our case *Fragilaria* and *Nitzschia*) only grew significantly in the Laguna de La Cruz in the autumn 1998, when the *Cosmarium bioculatum* population reached its highest values.

The autumn overturn in Laguna de La Cruz is not only due to wind-induced vertical mixing, but it is also strongly influenced by the horizontal circulation of convection currents from the littoral zone to the lake's centre (MacIntyre & Melack, 1995). These horizontal movements occur when nocturnal heat losses become very strong in late autumn, though a steep vertical density gradient is still present. Then, convective motions induced by thermal instabilities could be as important as wind mixing for the aeration and upwelling of deep water. This would partly support the growth peaks of the species with littoral affinities like those of the genus *Cosmarium* during this period. This is also shown by the interannual variations of autumnal phytoplankton development; e.g. in mid-late autumn 1998, a slightly shallower thermocline with a very steep density gradient greatly favoured blooms of *Cosmarium* species. Atelomixis, this is, partial mixing of the water column, has also a role in favouring *Cosmarium* species (Souza *et al.*, 2008), allowing that these non-motile species can reduce sinking thanks to the turbulence created by this partial mixing. This could also be important even for small species like *C. bioculatum*, which blooms under such conditions in Laguna de La Cruz.

The influence of grazing on phytoplankton species succession is evidenced in Laguna de La Cruz when observing the anti-predation features of phytoplankton species during the stratification period, when zooplankton densities are the highest, especially of rotifers and cladocerans of genus *Diaphanosma* and the species *Ceriodaphnia dubia*; (Boronat, 2003). For example, Mayeli *et al.* (2004), confirmed how both a larger colony size and spine formation in *Scenedesmus quadricauda* are effective defences against grazing by rotifers and small cladocera like *Ceriodaphnia dubia*. As the stratification period advances, larger sized phytoplankton species, which are mainly inedible for the dominating zooplankton, become more important in Laguna de La Cruz. Moreover, many small chlorophyceae that grow in this lake mostly during the stratification period present structures that are useful to avoid zooplankton grazing, but also contribute to slow down sinking. As such, *Lagerheimia* and *Scenedesmus* display spines, *Kirchneriella* and *Pseudoquadrigula* are surrounded by a mucilage, and in some *Cosmarium* species, e.g., *C. abbreviatum* and *C. bioculatum*, a thick mucilaginous cover can also be observed. All this supports a role of grazing avoidance, additionally to the main effects of vertical stratification, in shaping the phytoplankton assemblages in Laguna de La Cruz during the stratification period.

In the depths near the oxycline, when silica is still available, *Cyclotella* establishes first at the onset of the stratification, but it is progressively displaced by species that appear in spring in upper layers, such as those of the genera *Synechococcus*, *Rhodomonas* and *Cryptomonas*, which later on remain confined in the nutrient-rich oxycline stratum. These species of coda **Y**, **Z** and **W1**, which correspond to cryptophytes, picocyanobacteria and euglenophytes respectively, replace those of groups **B** and **Z** in deep layers during the stratification period. Nanophytoplankton succession no longer progresses in the oxycline, keeping the characteristic structure of a microbial plate established on the density gradient in the oxycline-redoxcline zone of such type of meromictic lakes (Zadereev, 2017). In Laguna de La Cruz, this is formed by a dense multilayer structure dominated, from top to the bottom of the oxycline, by picocyanobacteria accompanied with some species of chlorophytes, cryptophytes, and euglenophytes, overlying the anoxic layers where sulphur bacteria -Chromatiaceae and Chlorobiaceae- develop (Vicente & Miracle, 1988; Miracle *et al.*, 1992; Dasí & Miracle, 1991, Rodrigo *et al.*, 2000; Camacho *et al.*, 2017). In the upper zone of the oxycline, where nutrients are available, but where both oxygen concentrations and light availability are low, the dominant nanophytoplankton includes species of *Rhodomonas* and *Cryptomonas* which, as the picocyanobacteria, hold phycoerythrin as a photosynthetic adaptation to harvest light at low intensities, but mainly centered on a certain range of the spertrum that can be harvest by this pigment (Gervais, 1998, Camacho *et al.*, 2001, 2003a). The ability of harvesting the dim light reaching these depths, together with its mixotrophic capacity and motility, also explains the development of *Cryptomonas* spp. in the chemocline of Laguna de La Cruz (Camacho *et al.*, 2001).

Our data corroborate previous statements (Dasí & Miracle, 1991) that phytoplankton diversity in Laguna de La Cruz inversely correlates with cell abundance. This negative correlation is a general feature commonly found in many natural lakes, like the neighbouring Laguna del Tejo (Morata *et al.*, 2003), as well as in reservoirs (De León & Chalar, 2003). The lowest diversity was found when phytoplankton abundance peaked, with minimum values obtained in the two samplings when *Cyclotella delicatula* reached a relative abundance of up to 75 % along the water column. This is favoured by the absence of thermal gradients that differentially support environmental heterogeneity that could favour different algal groups at different depths. Contrastingly, higher diversity was found at the beginning and end of summer given the vertical heterogeneity of the water column produced by the thermal stratification, which explains the higher diversity when the water column was considered as a whole (Margalef, 1979; Tilman, 1994). However, in this period, drops in diversity were also observed, especially in the epilimnion, particularly those linked to the whitening event due to tumultuous calcium carbonate precipitation occurring every year around the end of July and the beginning of August. This causes a strong settling of phytoplankton and photosynthetic bacterial cells both by co-nucleation with calcite cristals and by the shadow effect of these cristals (Miracle *et al.*, 2000; Camacho *et al.*, 2003a; Romero-Viana *et al.*, 2006, 2011; Camacho *et al.*, 2017). The differential weather conditions in autumn 1998 (greater difference between the average maximum temperature and the average minimum temperature and the low rainfall compared to previous autumns) favoured greater stability compared to previous years, which comparatively enabled the development of higher biomass still maintaining a relatively high diversity in a heterogeneous water column (Fig. 6). The more intense, and slightly shallower, temperature gradient in the autumn of 1998, would explain the horizontal transport of nutrients and phytoplankton from the littoral zone to the pelagic area (MacIntyre & Melack, 1995).

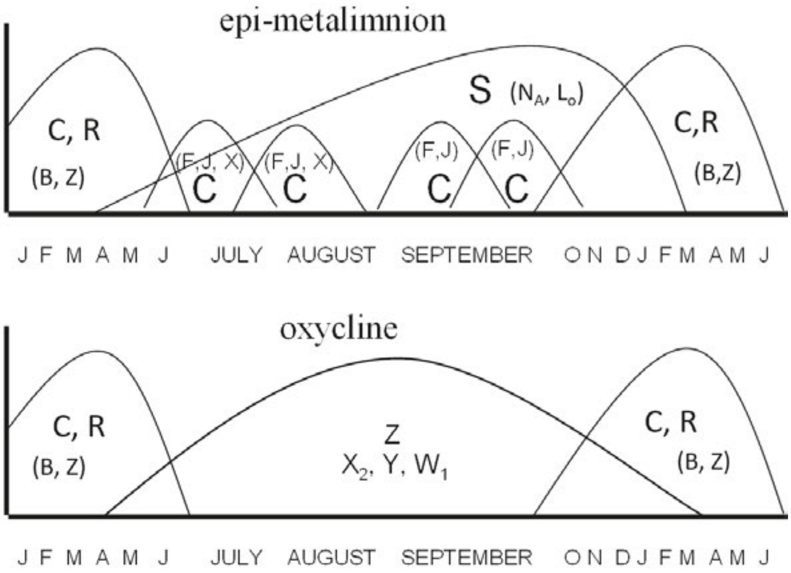


Figure 10. Phytoplankton annual sequence in the epi-metalimnion and the oxycline of Laguna de La Cruz according to the strategies proposed by Reynolds (1997), R, C and S strategists. *Secuencia anual del fitoplancton en el epi-metalimnion y la oxiclina de la Laguna de La Cruz de acuerdo con las estrategias propuestas por Reynolds (1997), estrategas R, C y S.*

Depending on the strategies of the different species, and following Reynolds (1997), the yearly phytoplankton sequence in the epilimnion-metalimnion of the Laguna de La Cruz (Fig. 10) partly coincides with the ideal sequence for a warm monomictic lake. However, in this lake, during stratification, there is no simple replacement of R-strategy species by C-strategy species followed by S-strategy species. Instead, there is also a replacement among C-strategists species when they dominate. As a consequence of the main disturbance, the whithening event, the autogenic succession pattern typically resulting from a period of stratification with stability is not well fitted. Whithening promotes a strong decrease in transparency, in such a way that most phytoplankton biomass can not be sustained and sinks, as demonstrated by the sedimentation patterns (Miracle *et al.*, 2000), as well as by the drop in biomass and chlorophyll-*a* concentrations following the whithening event (Fig. 5). This is a very severe disturbance that, in contrast with intermediate disturbances, causes a drop both in biomass and diversity as strongly affecting the whole community, and this explains the concomitant drop in diversity (Sommer *et al.*, 1993). This, together with other disturbances such as storms, is why diversity does not follow a simple increasing pattern with plankton's annual succession until autumnal mixing, but instead rises and falls during summer as a consequence of the differential effects of these disturbances (Naselli-Flores *et al.*, 2003). This is especially clear in the epilimnion, where these effects are stronger, as also documented for deep alpine lakes by Dokulil and Teubner (2003). Our work confirms that, as for other lakes (Padisák *et al.*, 1998; Becker *et al.*, 2010; Xiao, 2011; Martinet *et al.*, 2015) functional groups represent a useful way to describe the succession of phytoplankton.

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Meromictic lakes, methanogenic bacteria consortia

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Interspecies interactions mediated by conductive minerals in the sediments of the Iron rich Meromictic Lake La Cruz, Spain

Amelia-Elena Rotaru[1,*], Nicole R. Posth[2], Carolin R. Löscher[1,3], Maria R. Miracle[4,†], Eduardo Vicente[4], Raymond P. Cox[5], Jennifer Thompson[6], Simon W. Poulton[6] and Bo Thamdrup[1]

[1] Department of Biology, University of Southern Denmark, Campusvej 55, 5230 Odense, DK.
[2] Department of Geosciences and Natural Resource Management (IGN), Geology, University of Copenhagen, Øster Voldgade 10, 1350 Copenhagen, DK.
[3] Danish Institute for Advanced Science, University of Southern Denmark, Campusvej 55, 5230 Odense, DK.
[4] Instituto Cavanilles de Biodiversidad y Biología Evolutiva, University of Valencia, 46980 Paterna, Spain.
[5] Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, 5230 Odense, DK.
[6] School of Earth and Environment, University of Leeds, Leeds, LS2 9JT, UK.
[†] Deceased. This article is *in memoriam* of Professor Maria R. Miracle.

[*] Corresponding author: arotaru@biology.sdu.dk

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ABSTRACT

Interspecies interactions mediated by conductive minerals in the sediments of the Iron rich Meromictic Lake La Cruz, Spain

Lake La Cruz is considered a biogeochemical analogue to early Earth marine environments because its water column is depleted in sulfate, but rich in methane and iron, similar to conditions envisaged for much of the Precambrian. Here we show that conductive particles drove the metabolic coupling between electroactive microbial clades from this environment. The anoxic sediment of Lake La Cruz was rich in biogeochemically 'reactive' iron minerals, and harbored known electroactive species such as *Geobacter* and *Methanothrix*, in addition to groups never linked to an electroactive lifestyle. Slurry incubations on various substrates in the presence of conductive particles showed 2 to 4 times higher methanogenic activity, as compared to incubations with non-conductive glass beads or without added particles. In the absence of conductive particles, all tested substrates were metabolized to acetate, which accumulated above 8 mM depending on substrate (8±0.6 to 11.7±1.2 mM). Only by enabling syntrophic acetate oxidation with conductive minerals could we prevent acetate accumulation. Acetate oxidation conductively coupled to methanogenic activity had a stoichiometric recovery of 70 % and could be maintained in subsequent transfers only if amended with conductive particles. Mud-free enrichments without conductive particles ceased any metabolic activity after the second transfer. Conductive particles preserved a consortium of *Youngiibacter-Methanothrix*, whereas without conductive particles *Youngiibacter* spp. died off. Syntrophic consortia from this early Earth analogue environment only survived in the presence of conductive particles inferring that minerals may have arbitrated the earliest interspecies associations.

Key words: *Methanothrix*, *Geobacter*, *Youngiibacter*, magnetite, conductive particles, granular activated carbon, direct interspecies electron transfer, mineral mediated syntrophy, iron meromictic lake

RESUMEN

Interacciones interespecíficas mediadas por minerales conductivos en el sedimento del Lago La Cruz (España), un lago meromíctico rico en hierro

La laguna de La Cruz puede considerarse como un análogo biogeoquímico de los ambientes marinos en la Tierra primitiva, porque su columna de agua, muy escasa en sulfato pero rica en metano y hierro, presenta condiciones similares a estos ambientes en el Precámbrico. En este ambiente encontramos que partículas conductivas establecen un acoplamiento metabólico *entre clados microbianos electroactivos. Demostraremos que el sedimento anóxico de la laguna de La Cruz, rico en minerales de hierro biogeoquímicamente "reactivos", alberga conocidas especies electroactivas tales como* Geobacter *y* Methanothrix*, además de otros grupos microbianos que no han sido previamente asociados con un estilo de vida electroactivo. Las incubaciones de lodo en varios sustratos realizadas en presencia de partículas conductivas tuvieron como resultado una actividad metanogénica de dos a cuatro veces mayor, comparadas con las incubaciones con microperlas de vidrio no conductivas o sin partículas adicionadas. En ausencia de partículas conductivas, todos los sustratos probados fueron metabolizados a acetato, que se acumulaba hasta una concentración de hasta aproximadamente 8 mM dependiendo del sustrato (8±0.6 to 11.7±1.2 mM). Sólo la adición de mineral conductivo a los lodos de la laguna de La Cruz hacía posible la oxidación del acetato, impidiendo su acumulación. La oxidación del acetato acoplada a una alta actividad metanogénica tuvo una recuperación estequiométrica del 70 % y pudo mantenerse en sucesivos enriquecimientos libres de sedimento solo si estos se adicionaban con minerales conductivos. Por el contrario, enriquecimientos libres de sedimento y sin partículas conductivas cesaron cualquier actividad metabólica en la segunda transferencia de la serie. Las partículas conductivas conservaron la existencia del consorcio* Youngiibacter-Methanothrix*, mientras que* Youngiibacter *spp. muere y desaparece en ausencia de las partículas conductivas. Consorcios sintróficos de este ambiente análogo al de la Tierra primitiva sólo sobrevivieron en presencia de partículas conductivas infiriendo que los minerales pueden haber mediado en las asociaciones interespecíficas más primitivas.*

Palabras clave: Methanothrix*,* Geobacter*,* Youngiibacter*, magnetita, partículas conductivas, carbón activo granulado, transferencia directa de electrones interespecífica,sintrofia con mediación mineral, lago meromíctico ferruginoso*

INTRODUCTION

It has been proposed that eukaryotic life arose from syntrophic interactions between Deltaproteobacteria and methanogenic archaea (Moreira & Lopez-Garcia, 1998; López-García & Moreira, 1999; Martin & Russell, 2003) in the anoxic and ferruginous (Fe-rich) early Archaean ocean (Crowe *et al.*, 2008). Similar conditions exist today in the anoxic deeper waters of some lakes (Crowe *et al.* 2008; Bura-Nakic *et al.*, 2009; Llirós *et al.*, 2015), including Lake La Cruz, Spain (Walter *et al.*, 2014; Camacho *et al.*, 2017). Most studies of these environments have focused on the phototrophic and methanotrophic communities in the water column, yet little attention has been given to either the methanogenic community buried in the sediments or the possible impact of iron-minerals on their physiology. Only recently, have researchers investigated the methanogenic community from Lake Matano, Indonesia which displayed high methanogenic rates when spiked with the iron-oxide, goethite (Bray *et al.*, 2017). However, the possibility of a mineral-mediated syntrophic interaction was not assessed.

Generally, syntrophic associations are carried out indirectly, in which case electron transfer between partners is assisted by diffusible chemicals (H_2, formate, shuttles). These classical syntrophic interactions require a bacterium and a methanogenic archaeon as syntrophic partner. The bacterium oxidizes complex organics to reduced compounds (i.e. H_2), that are then retrieved by the methanogenic archaeon in order to reduce CO_2 to methane (Shrestha & Rotaru, 2014). Recent studies have shown that, sometimes, interspecies electron transfer does not require a diffusible chemical carrier. In the absence of a diffusible electron carrier, interspecies electron transfer could occur via conductive particles (magnetite, chars, pyrite) (Liu *et al.*, 2012; Chen *et al.*, 2014; Liu *et al.*, 2015; Kato & Igarashi, 2018; Wang *et al.*, 2018) or directly by forging electric connections via a self-assembled extracellular network of conductive pili and *c*-type cytochromes between the two syntrophic partners (Summers *et al.*, 2010; Rotaru *et al.*, 2014b, 2014a). The later is known as direct interspecies electron transfer (DIET). DIET was shown to be accelerated by conductive materials possibly because cells save energy by pausing the production of their own conductive extracellular network (Liu *et al.*, 2015; Wang *et al.*, 2018). Consequently, mineral-mediated syntrophy is energetically more favorable than the usual syntrophic associations.

It has been proposed that Fe-minerals such as pyrite helped nucleate the membranes of the earliest cells (Wächtershäuser, 1988a; Russell *et al.*, 1994). Many membrane bound proteins involved in electron transfer through the membranes of present day cells contain FeS centers (i.e. ferredoxins). It is therefore likely that some of the earliest FeS proteins might have played a role in electron transfer between cells.

It has been speculated that conductive-minerals also mediate the interaction between protocells with leaky cell walls present in the mineral-rich Archaean ocean (Lane & Martin, 2012). Interactions between cells with different metabolisms are thought to be at the origin of eukaryogenesis, as such cells compartmentalized the functions within the eukaryotic cell (Moreira & Lopez-Garcia, 1998; López-García & Moreira, 1999; Martin & Russell, 2003). In the present study, we investigated the conductive iron-mineral dependency of interspecies interactions between bacteria and methanogens from the sediments of the Fe-rich, stratified Lake La Cruz. Specifically, we were interested in whether reactive Fe minerals would support conductive-mineral mediated interspecies interactions. As the biogeochemical setting of the lake makes it a prime early analogue (Walter *et al.*, 2014, Camacho *et al.*, 2017), we also discuss mineral-mediated syntrophy as a relic of ancestral associations.

MATERIAL AND METHODS

Sampling and incubations

During an expedition at Lake La Cruz in central Spain (Fig. 1) in September 2014, we sampled the lake water and sediment. Lake La Cruz is a permanently stratified, meromictic, doline lake located in a karst region in the Iberian Mountain Range. The lake is circular with a diameter of 122 m. At the time of sampling, the maximum depth was 20 m and the chemocline started at ~12 m depth. Water samples were pumped from depths above,

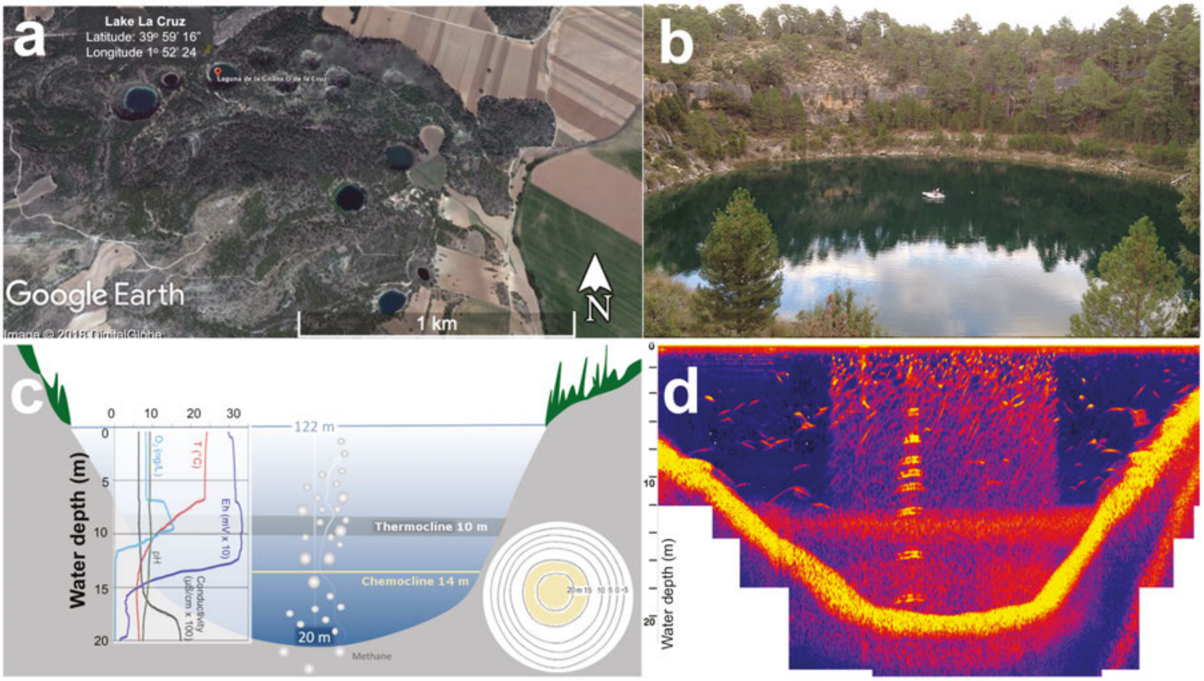

Figure 1. Lake La Cruz with its geophysical features. Map of the Cuenca lake area with geographical coordinates (a); and an image of the sampling site - lake La Cruz (b). Schematic representation of the lake (c) including a bathymetric map (c-round inset), and *in situ* physico-chemical characteristics of the water column (c-graphic inset: ▬ conductivity µS/cm, ▬ temperature ºC, ▬ oxygen mg/L, ▬ pH, ▬ Eh mV). An echogram indicating the chemocline from 12-14 m, with visible gas ebullition in the central area (d). *La laguna de La Cruz con sus características geofísicas. Mapa de la zona de Cuenca con coordenadas geográficas del lago estudiado (a); y una imagen del lugar de muestreo – laguna de La Cruz (b). Representación esquemática del lago (c) que incluye un mapa batimétrico y las características físico-químicas* in situ *en la columna de agua: (▬ conductividad µS/cm, ▬ temperatura ºC, ▬ oxígeno mg/L, ▬ pH, ▬ Eh mV). El ecograma muestra la quimioclina situada a 12-14 m, con ebullición gaseosa visible en la zona central (d).*

within, and below the chemocline at the deepest part of the lake from a boat tethered from shore to shore of the lake. The pumping apparatus was designed to withdraw water samples without contact with the atmosphere, and both the apparatus and sampling protocol have previously been described in detail (Miracle *et al.*, 1992; Posth *et al.*, 2017). Samples were gathered and fixed directly on the boat and stored until analysis in the lab.

Three sediment cores were collected from the center and the deepest part of the lake (coordinates: 1° 52' 24" West; 39° 59' 16" North, Fig 1) using a sediment corer (Kajak sediment core, KC Denmark). The cores (50 cm length × 7 cm diameter) were sealed without air bubbles as they were pulled up from depth with rubber stoppers immediately inserted to avoid exposure to the atmosphere. Within 24 hours of sampling, the sediment was partitioned into depth intervals, and fixed for biogeochemical and molecular analyses inside an N_2-filled inflatable glove bag, as described in detail below.

For downstream incubations, sediment from 0-15 cm depth was sampled and placed in Duran bottles secured with butyl-rubber stoppers, with a headspace of 2 bars N_2:CO_2 80:20 mix. Samples were stored at 4 °C until later used for incubations.

Slurries were prepared in an N_2-filled anaerobic chamber in the laboratory. For these slurries, we used 3 mL cut-off syringes to distribute 2.5 mL of sediment into 20 mL gas-tight vials filled with 7.5 mL of medium, either modified DSM 120 or DSM 334. Modified DSM 120 medium was prepared as described previously (Rotaru *et al.*, 2014b), but with 0.6 g/L NaCl. Three successive ten-fold dilutions of the sediment slurries led to essentially mud-free enrichments in which sediment particles could not be detected visually or by microscopy. Before inoculation, the complete medium, which lacked the substrate and (semi)conductive minerals, was dispensed anaerobically by syringe into sterile degassed vials with or without minerals prepared as below.

Two electrically conductive particle types (granular activated carbon and magnetite) were selected to be tested because they were previously confirmed to stimulate DIET in methanogenic co-cultures (Liu *et al.*, 2012; Zheng *et al.*, 2017). Granular activated carbon (GAC, Sigma Aldrich) had a particle size between 180 and 300 µm diameter and estimated conductivity of circa 1000 S/m (Kastening *et al.*, 1997), and magnetite (Sigma Aldrich) with particles less than 5 µM diameter, and estimated electrical conductivity ranging between 0.1 and 1 S/m (Rochelle & Schwertmann, 2003; Blaney, 2012). Both materials have conductivities similar or higher than the pili that carry out extracellular electron transfer in *Geobacter sulfurreducens* (5 S/m (Adhikari *et al.*, 2016)). We weighed 0.1 g/L of each material, added to vials, overlaid with 200 µl ultrapure water for wet sterilization, degassed for 3 minutes with N_2:CO_2 80:20 mix, and autoclaved at 121 °C for 25 min. Controls with non-conductive particles were setup with acid-washed glass beads (less than 105 µm diameter) instead of conductive minerals. Substrates (5 mM glucose, 5 mM sodium butyrate, 10 mM sodium acetate, 10 mM ethanol) were added to media from sterile anoxic 1 M stocks using aseptic and anaerobic techniques. Controls without electron donors were carried out in order to identify whether the organics in the sediment served as substrates for methanogenesis. All incubations were carried out at room temperature (20-23 °C) in triplicate, unless otherwise stated.

Gas samples were withdrawn, stored anaerobically and then analyzed for methane on a Thermo Scientific gas chromatograph (Rotaru *et al.*, 2018). To test for short chain volatile fatty acids (SCVFA) we used high performance liquid chromatography (HPLC) as described elsewhere (Rotaru *et al.*, 2018).

Biogeochemistry

For biogeochemical parameters, we took water column samples at different depths and sampled the sediment obtained via the gravity corer. Geochemical parameters of relevance to this work were methane, soluble ferrous iron, and particulate reactive iron mineral species. We will use the term reactive iron species to refer to oxalate, dithionite and HCl soluble iron oxides and sulfides (Poulton *et al.*, 2004).

Water column methane was sampled from the pumping apparatus through chemically resistant tubing (VWRs ISO-VERSNIC®) into 20 mL glass, GC vials (Supelco, Sigma-Aldrich). For each sampling depth, 5 mL samples in triplicate were added to GC vials pre-doped with 10 mL 2 N NaOH to retain CO_2 in the liquid phase. The vials were sealed with butyl-viton rubber stoppers, and stored upside down in the dark at 4 ˚C until analysis.

Sediment methane concentrations were determined from sediment slices extracted every 2 cm in an anoxic glove bag filled with N_2 gas.

Our measurements of available electron acceptors at the sediment boundary layer corroborated previous investigations during summer months at this lake (Miracle *et al.*, 1992; Walter *et al.*, 2014; Camacho *et al.*, 2017) and showed a depletion of sulfate and Fe^{3+} (< 10 µM sulfate, < 1 µM Fe^{3+}) and no detection of oxygen and nitrate. Thus sediments mainly relied on methanogenesis for decomposition of organic matter below the water-sediment boundary. For sedimentary methane determination, sliced sediment was filled into glass GC vials, to which 1 M (2.5 %) NaOH was added in order to stop any additional microbial activity. The vials were capped with butyl-viton stoppers, crimped, and inverted until lab analysis. Sedimentary methane concentrations were determined on a Perkin Elmer GC, as previously described (Rotaru *et al.*, 2018).

Porewater was analysed for reduced iron concentrations at ~2 cm depth resolution after extraction using Rhizons (Rhizosphere; pore size 0.2 µm) inside a glove bag with an N_2-atmosphere. Dissolved Fe^{2+} was determined immediately using the ferrozine assay (Lovley & Phillips, 1987; Viollier *et al.*, 2000).

To determine iron mineral speciation, sediment was subsampled at each 2 cm-depth interval and stored at -20 °C. Reactive iron species (dithionite and HCl soluble iron species) were identified from freeze-dried samples stored at -20 °C by applying a modified sequential iron extraction procedure (Poulton & Canfield, 2005). In the first step, a room temperature 0.5 N HCl extraction was applied to dissolve poorly crystalline iron oxides such as ferrihydrite, surface absorbed Fe^{2+}, iron carbonate minerals such as siderite, and acid volatile iron monosulfides (Zegeye *et al.*, 2012). Subsequently, a pH 4.8 sodium dithionite extraction was employed to dissolve crystalline ferric oxide minerals such as goethite and hematite, followed by an oxalate extraction to dissolve magnetite (Poulton & Canfield, 2005). The total concentration of iron dissolved in each operationally defined extraction phase was determined by flame atomic absorption spectroscopy (AAS). For the 0.5 N HCl extraction, dissolved Fe^{2+} was also measured immediately via the ferrozine assay (Lovley & Phillips, 1987). Extraction of this Fe^{2+} from the total Fe determined for this extraction by AAS gave the Fe^{3+} concentration associated with poorly crystalline iron oxides such as ferrihydrite. Iron sulfide phases were determined via a two-step sequential extraction procedure (Canfield *et al.*, 1986). Acid volatile sulfide minerals (FeS) were determined by extraction with hot 6 N HCl under N_2, with the released sulfide trapped as Ag_2S. Pyrite (FeS_2) was then determined after addition of chromous chloride, with the sulfide again trapped separately as Ag_2S. After filtration, the concentrations of Fe in FeS and FeS_2 were determined stoichiometrically. The concentration of Fe present as FeS was subtracted from the Fe^{2+} concentration determined by the 0.5 N HCl extractions, to give surface reduced and carbonate-associated Fe^{2+}. Replicate extractions gave a RSD of < 5 % for all phases.

Scanning electron microscopy

Samples from the water column were preserved in 4 % formalin, filtered on Nucleopore carbonate filters, with a pore size of 0.2 µm, and dehydrated in 20 min. steps with ethyl alcohol (30 %, 50 %, 70 %, 90 % and two times 100 %). Then the samples were critical point dried prior to palladium/gold sputter coating and visualization on a Hitachi S-4800 FE scanning electron microscope ran at an electron beam acceleration voltage of 20 kV.

Molecular analyses

For molecular analyses we sampled 2 mL of sediment at 2 cm depth resolution using cut-off syringes inside a N_2-filled glove bag. Sediment was pooled together every 4 cm and fixed with MoBio RNAlater 1:1 v/v (Rotaru *et al.*, 2018).

Prior to DNA extractions, most of the RNAlater was removed by centrifugation. For DNA extraction we used the top 16 cm of sediment from triplicate cores. Extractions were carried independently for each core with the MoBio RNA Soil kit coupled to the MoBio complementary DNA Soil kit, following the manufacturer's protocol. DNA was quantified using a Nano Drop prior to downstream applications. The DNA extracted from each core was amplified with the following primer pair S-D-Arch-0519-a-S-15/S-D-Bact-0785-b-A-18, which according to Klindworth *et al.* (2013), was the best for MiSeq amplicon sequencing, targeting more than 89 % of Bacteria and more than 88 % of Archaea. PCR amplification and indexing (using Nextera XT index kit, Illumina) of the PCR products for the triplicate samples was conducted following the Illumina 16S rRNA gene amplicon sequencing protocol (Illumina, USA). The DNA samples were then sequenced using ×300 PE MiSeq sequencing approach at Macrogen (www.macrogen.com), using Illumina's protocol. The sequences generated circa 1 million reads for each core, which were imported into CD-HIT-OTU to remove noisy data and clustered into OTUs, using a 97 % species

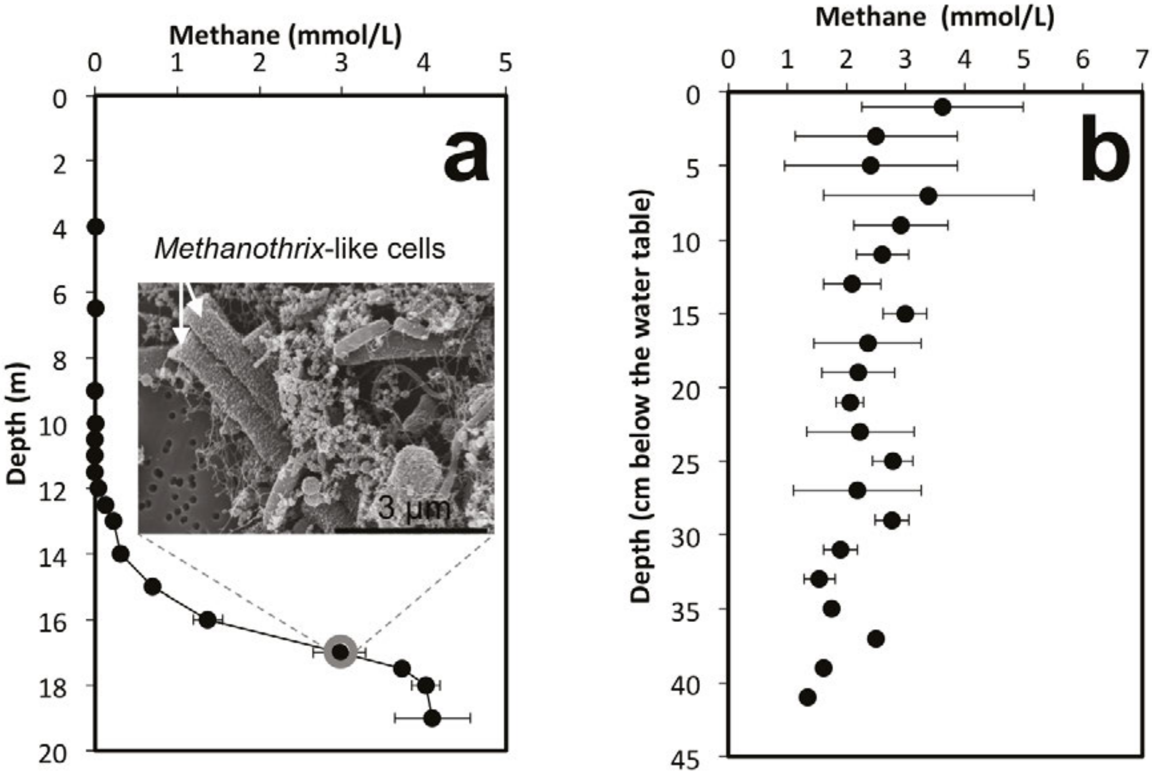

Figure 2. Methane profiling through the water column and sediment of lake La Cruz. (a) In the water column of Lake La Cruz, the highest methane concentrations were below 17-m depth where *Methanothrix*-like cells (inset) could be observed by scanning electron microscopy. (b) Sediment cores showed very high methane concentrations especially in the top 15-cm, indicating that methane has also sedimentary origin. The water column values as average ± standard error values are for triplicate samples taken at each specific water column depth; while values for sediments are from triplicate cores sampled every 2-cm. *Perfiles de metano en la columna de agua y en el perfil de los sedimentos de la laguna de La Cruz. (a) En la columna de agua de la laguna de La Cruz, las concentraciones más altas de metano se encontraban por debajo de los 17 m de profundidad, donde células similares a* Methanothrix *(recuadro) se podían observar mediante microscopía electrónica de barrido. b) Los testigos de sedimento mostraron concentraciones muy elevadas de metano, especialmente en los 15 cm superiores, lo que indica que el metano también tiene su origen en el sedimento. Los valores en la columna de agua son la media ± el error estándar de muestras triplicadas tomadas a cada profundidad específica en la columna de agua; mientras que los valores en los sedimentos son la media de testigos triplicados muestreados cada 2 cm.*

cutoff. For taxonomy and diversity analyses, clean and clustered OTUs were analyzed using QIIME (Caporaso *et al.*, 2010), against the Ribosomal Database Project database version 11. Alpha rarefaction analyses showed sufficient coverage of the diversity in all three sediment cores.

DNA extractions from mud-free incubations were performed using the MasterPure DNA purification kit as previously described (Rotaru et al., 2014b). Amplification of bacterial (27F, 5′-AGAGTTTGATCMTGGCTCAG and 1492R, 5′-TACCTTGTTACGACTT) and archaeal (344F – 5’-ACGGGGYGCAGCAGGCGCGA -3’ and 1059R – 5’- GCCATGCACCWCCTCT-3’) 16S rDNA sequences, library preparation, and 16S rRNA gene sequencing, was performed as previously described (Rotaru *et al.*, 2018). Maximum likelihood phylogenetic trees were constructed using Geneious (Kearse *et al.*, 2012). Sequence files can be found at NCBI under Bioproject ID: PRJNA510210.

RESULTS AND DISCUSSION

Our hypothesis was that the iron-rich Lake La Cruz would be a breeding ground for conductive, mineral-based syntrophy (Rotaru *et al.*, 2018). We discovered that microorganisms enriched from Lake La Cruz carried out syntrophic degradation strictly dependent on conductive mineral additions and were unable to carry unaided DIET associations.

Geochemistry

We expected to find a niche for DIET/conductive-particle mediated IET in this Fe-rich methanogenic lake resembling the ocean in the Precambrian. La Cruz sediments displayed high methane concentrations in the top 15 cm, along with a significant proportion of reactive iron species ~70 % of the total Fe content) (Thompson 2018), which is very high relative to normal non-ferruginous aquatic environments (Poulton & Raiswell, 2002). During this sampling campaign, the sediments were overlain by ~10 m of anoxic water (Fig. 1). During summer months, the lake is known to persistently have a 4-5 m monimolimnion zone above the sediment, which is rich in Fe^{2+} (Vicente & Miracle, 1988). In our study we also noticed strong methane super-saturation near the bottom, where the methane concentration reached 4 mM, similar to concentrations in the surface sediment (Fig. 2). Gas ebullition was observed during sampling from the deep water table. Oftentimes, gas bubbles mainly consisting of methane and carbon dioxide (Camacho *et al.*, 2017), percolated through the surface of the lake from the middle, as documented by an ecogram of the lake (Fig. 1d). Previous studies suggest that the sediment is the source of water-column methane (Oswald *et al.*, 2016). Indeed we observed that sediment methane concentrations were highest in the top centimeters of the sediment (Fig. 2). Methane concentrations were also high in the water column (17-20 m), indicating methanogenesis occured in the bottom waters as well as the top layers of the sediment (Fig. 2).

As documented previously (Oswald *et al.*, 2016; Camacho *et al.*, 2017), dissolved Fe^{2+} did build up below the chemocline to reach concentrations of ~250 µM above the sediment-water interface. In the sediment, dissolved Fe^{2+} concentrations continued to increase (Thompsen, 2018), reaching a peak of > 1000 µM at a depth of ~22 cm. These high dissolved Fe^{2+} concentrations in the water column and sediment porewaters are similar to those found in other iron-rich lakes (Vicente & Miracle, 1988; Bura-Nakic *et al.*, 2009; Crowe *et al.*, 2011; Nordi *et al.*, 2013) . The La Cruz sediments were high in TOC (average = 6.68±2.0 wt %), and carbonate minerals (average = 9.46±1.3 wt % inorganic C), which diluted the total Fe-content to 1.06±0.18 wt % on average (Thompson, 2018). This is considerably lower than the average global total Fe content of riverine particulates supplied to oceans and lakes (4.49 wt %; Poulton & Raiswell, 2002). Proportionally, however ‘reactive’ Fe phases (non-sulphidized Fe^{2+}, Fe-oxides, Fe-sulfides) were abundant (70±8 %; Thompson, 2018) relative to the total Fe content of the sediment, of which only 18±5 % was sulfur bound (pyrite, other Fe-S minerals). Nevertheless, magnetite Fe accounted for less than 0.1 % of the total Fe-content in this sediment, while other Fe oxide minerals accounted for ~10 % of total Fe on average. Thus, non-sulphidized particulate Fe(II) was the dominant reactive Fe pool (~60 % on average).

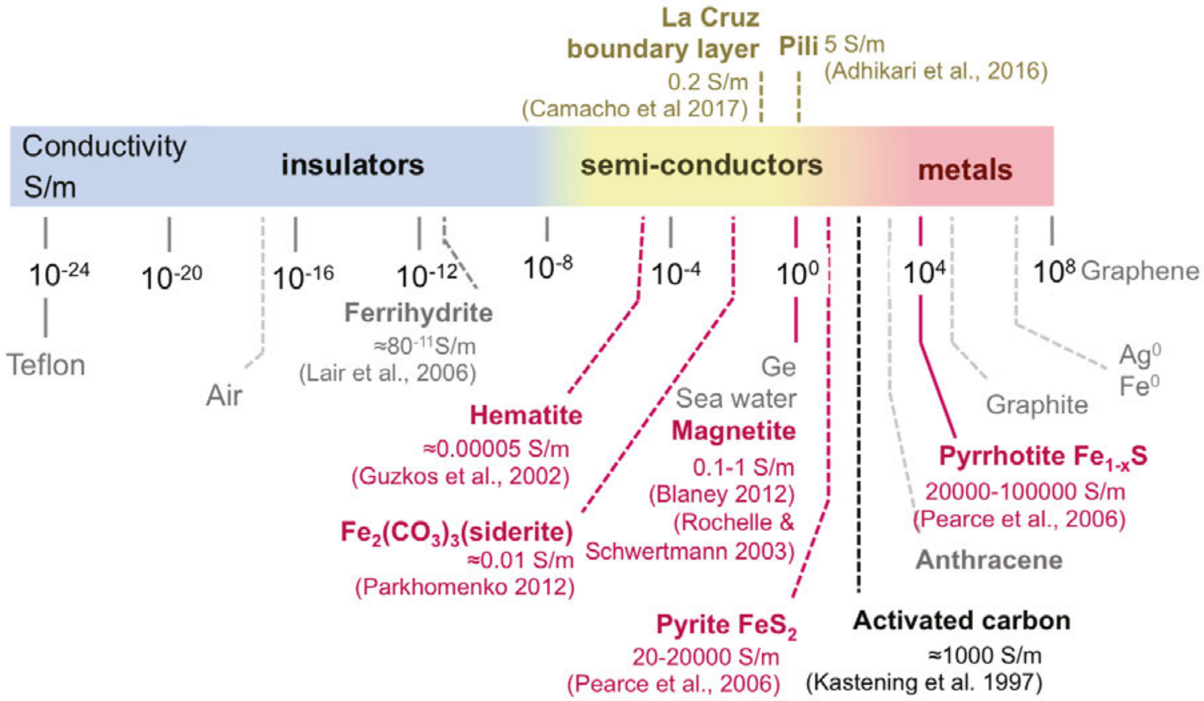

Figure 3. Conductivities of various Fe-oxides and Fe-sulfides, compared to that of the e-pili and the conductivities observed for different carbon particles including activated carbon used in this study. We have also listed the conductivity measured in lake La Cruz at the sediment-water boundary layer. (Adhikari *et al.*, 2016; Blaney, 2012; Camacho *et al.*, 2017; Guskos *et al.*, 2002; Kastening *et al.*, 1997; Lair *et al.*, 2006; Parkhomenko, 1990; Pearce *et al.*, 2006; Rochelle and Schwertmann, 2003). *Conductividades de varios óxidos y sulfuros de Fe, comparadas con las de los pelos bacterianos conductores (e-pili) y las conductividades observadas para diferentes partículas de carbón incluyendo el carbón activado utilizado en este estudio. También hemos enumerado la conductividad medida en la laguna de La Cruz en la interfase sedimento-agua. (Adhikari* et al.*, 2016; Blaney, 2012; Camacho* et al.*, 2017; Guskos* et al.*, 2002; Kastening* et al.*, 1997; Lair* et al.*, 2006; Parkhomenko, 1990; Pearce* et al.*, 2006; Rochelle & Schwertmann, 2003).*

Some of the iron minerals (Fe-sulfides and Fe-oxides) found in the sediments of lake La Cruz are electrically conductive (Fig. 3), of which magnetite and iron sulfides have been documented to facilitate mineral mediated syntrophy (Liu *et al.*, 2012, 2015; Zheng *et al.*, 2017; Rotaru *et al.*, 2018; Kato & Igarashi, 2018). Fe-sulfides, like pyrite have been also shown to aid long-range extracellular electron transfer from cells (Kondo *et al.*, 2015) or enzymes (Mahadevan & Fernando, 2018) to electrodes. Moreover La Cruz sediments also contain coal particles (Romero-Viana *et al.*, 2011), which are conductive (Fig. 3). Indeed it has been documented that conductive carbon materials (i.e. granular activated carbon) facilitated mineral mediated syntrophy as effectively as conductive Fe-minerals (Liu *et al.*, 2012; Rotaru *et al.*, 2018).

In situ bacterial diversity – with focus on described electrogens

We therefore anticipated that electrically conductive particles inherent to La Cruz sediments would facilitate mineral mediated interactions between electrogens and electrotrophic methanogens. Indeed, our data demonstrate that the community harbors organisms affiliated to groups of electrogens including *Geobacter* (Fig. 4), and to DIET-methanogens including *Methanothrix* (Fig. 4). *Geobacter* and *Methanothrix* have previously been shown to carry out DIET in laboratory co-cultures (Rotaru *et al.*, 2014a), and have been found to co-exist in several man-made environments, such as rice paddies (Holmes *et al.*, 2017) and anaerobic digesters (Morita *et al.*, 2011; Rotaru *et al.*, 2014a). Our data showed that bacteria affiliated to known electrogens/iron-reducers like *Geobacter* (0.6 % of all Bacteria), *Thiobacillus* (0.2 % of all Bacteria), *Desulfobacterium* (0.4 % of all Bacteria), and *Anaerolinea* (0.1 % of all Bacteria) co-existed with *Methanothrix* in Lake La Cruz sediments (Fig. 4). Together, all of these putative electrogens/iron reducers, summed up to circa 1 % of all Bacteria. Previously, members of these four genera, *Geobacter*, *Thiobacillus*, *Desulfobacterium*, *Anaerolinea*, have been shown to be capable of extracellular electron transfer to and/or from electrodes or metallic iron (Nakasono *et al.*, 1997; Dinh *et al.*, 2004; Gregory *et al.*, 2004; Kawaichi *et al.*, 2018; Pous *et al.*, 2014; Rotaru *et al.*, 2015), as well as iron-minerals (Bosch *et al.*, 2012; Kawaichi *et al.*, 2013; Lovley *et al.*, 1993; Rotaru *et al.*, 2015). The first two, *Geobacter* and *Thiobacillus* can also interact by DIET with other cells (Summers *et al.*, 2010; Kato *et al.*, 2012; Rotaru *et al.*, 2014b, 2014a;), and this interaction has been shown to be expedited in the presence of conductive particles (Kato *et al.*, 2012; Liu *et al.*, 2012; Chen *et al.*, 2014;, Liu *et al.*, 2015; Rotaru *et al.*, 2014b; Zheng *et al.*, 2017). It is therefore possible that all of these electrogenic species compete for the electron uptake of electrotrophic methanogens.

However, one of the most abundant genera in these sediments was *Smithella* (2.6 % of all bacteria), which has been assumed to be electroactive and carry DIET interactions with *Methanothrix* in an alkane-degrading consortium (Embree *et al.*, 2014). Therefore, *Smithella* may establish a DIET-association with *Methanothrix* from Lake La Cruz sediments (see Archaea community below). Members of *Verrucomicrobia*

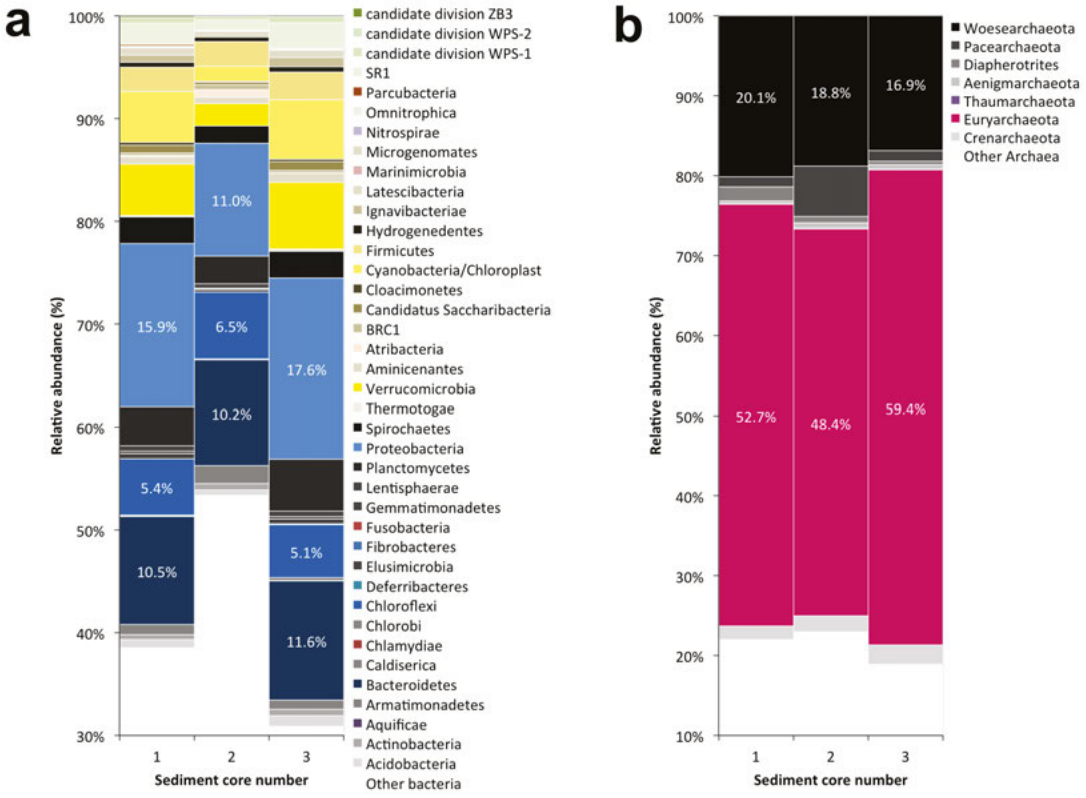

Figure 4. Relative phylum-level composition of (a) Bacteria and (b) Archaea harboring the top 16-cm of three sediment cores from lake La Cruz, as determined by 16S rRNA gene amplicon sequencing. *Composición relativa a nivel de filum de (a) Bacterias y (b) Arqueas albergadas en los 16 cm superiores de tres testigos sedimentarios de la laguna de La Cruz, según lo determinado por la secuencia del amplicón del gen 16S rRNA.*

were also very well represented (ca. 4.6 % of all bacteria) similar to what has been observed for 90 % of several lake sediments (He *et al.*, 2017). *Verrucomicrobia* were recently proposed to carry extracellular electron transfer due to their genetic make-up, which comprises the appropriate porin systems and membrane-associated *c*-type cytochromes (He *et al.*, 2017). It is also possible that *Verrucomicrobia* play a role in mineral mediated interspecies interactions. Nevertheless, *Verrucomicrobia* have never been shown to have the ability to interact syntrophically or to carry out extracellular electron transfer in laboratory cultures, and thus this predicted physiology requires further investigation. Some of the most abundant phyla were Bacteroidetes (10.8 % of the bacteria), and Firmicutes (2.5 % of all bacteria) (Fig. 4).

***In situ* archaeal diversity**

Euryarchaeaota accounted for more than half of the *Archaea* represented through amplicon sequencing (Fig. 4). Here, we show that in the sediments of Lake La Cruz, *Methanothrix* co-existed with electrogens (*Geobacter*, *Thiobacillus*, *Desulfobacterium*, and *Smithella*). Besides the acetoclastic/DIET-associated *Methanothrix* (3.7 % of all archaea), we identified canonical hydrogenotrophic-methanogens belonging to *Methanoregula* (2.5 % of all archaea), and very low numbers of *Methanobacterium* (0.2 % of all *Archaea*). The most abundant *Archaea* were the deep-branching *Methanomassilicoccus* (40.6 % of all archaea). The role of *Methanomassillicoccus* in sedimentary methanogenesis is not well understood since their only cultivated species-representative, *M. luminyiensis*, is a human-gut isolate strictly capable of H_2-dependent methylotrophic methanogenesis, but incapable of CO_2-reductive methanogenesis or acetoclastic methanogenesis (Dridi *et al.*, 2012a). Besides their documented presence in the gut of humans (Dridi *et al.*, 2012b), other animals (i.e. Salgado-Flores *et al.*, 2016) or insects (Paul *et al.*, 2012), *Methanomassilicoccus* sp. have also been found in anaerobic digesters (e.g. Chojnacka *et al.*, 2015), hydrothermal springs (e.g. Coman *et al.*, 2013), subsurface aquifers and soils (e.g. Kadnikov *et al.*, 2017), and aquatic sediments (e.g. Rotaru *et al.*, 2018) to name a few. *Methanomassiliicoccus* was also one of the most abundant genera of methanogens, not only in the iron-rich sediments of Lake La Cruz, but also in Baltic Sea sediments that are potential niches for conductive particle-mediated syntrophy (Rotaru *et al.*, 2018). It is possible that *Methanomassilicoccus* is involved in electroactive interactions via minerals, especially taking into account that this group was recently associated with electroactive communities abundant on electrodes from bioelectrochemical systems set up with inoculums from soils (Ahn *et al.*, 2014) and anaerobic digester sludge (Park *et al.*, 2018).

Among the methanogens detected in La Cruz sediments, only species of *Methanothrix* have been previously shown to establish DIET-associations with *Geobacter* species (Morita *et al.*, 2011; Rotaru *et al.*, 2014a; Wang *et al.*, 2016; Holmes *et al.*, 2017). *Methanothrix* was earlier suggested to carry out DIET with *Smithella* (Embree *et al.*, 2014), but the latter has never been shown to be capable of mineral-mediated or direct electron transfer. In a previous study, we have shown that a *Methanothrix*-species from the Baltic did not establish a mineral-mediated interaction with Baltic-*Geobacter*, but were instead competitively excluded by a *Methanosarcina-Geobacter* consortium, which carried a mineral-mediated syntrophic association (Rotaru *et al.*, 2018). However, although *Methanosarcina* is a very effective DIET partner (Rotaru *et al.*, 2014b, 2015) and mineral-syntrophy partner (Liu *et al.*, 2012; Chen *et al.*, 2014; Wang *et al.*, 2018; Rotaru *et al.*, 2018) they were poorly represented in La Cruz sediments (Fig. 4).

High methanogenic activity could only be maintained by conductive particles

To determine the effect of conductive particles on the Lake La Cruz methanogenic community, we compared incubations with or without additional conductive particles. These incubations showed that the methanogenic community was strictly dependent on the addition of conductive particles and independent of the type of substrate, conductive particle, or freshwater medium tested (Fig. 5). Incubations with conductive particles showed 2 to

4 fold increases in methanogenic rates (0.2-0.7 mM/day, depending on substrate) over incubations with non-conductive glass beads or without particle-amendment (0.09 to 0.18 mM/day, depending on the substrate). Moreover, high methanogenic activity was maintained in subsequent incubations *only if* conductive particles were added (Fig. 5). Cultures without conductive particles could not sustain methanogenesis for more than one subsequent transfer. This indicates a strict dependency of the enriched methanogenic community on conductive particles.

We observed that all tested substrates were transiently converted to acetate, which was converted quickly to methane in the presence of conductive particles, whereas acetate accumulated in the absence of conductive particles (Fig. 5). This is likely due to higher rates of acetate oxidation prompted by the addition of conductive particles, similar to previous observations of Bothnian Bay sediments where syntrophic acetate oxidation (SAO) relied on conductive minerals (Rotaru *et al.*, 2018).

We determined which organisms were

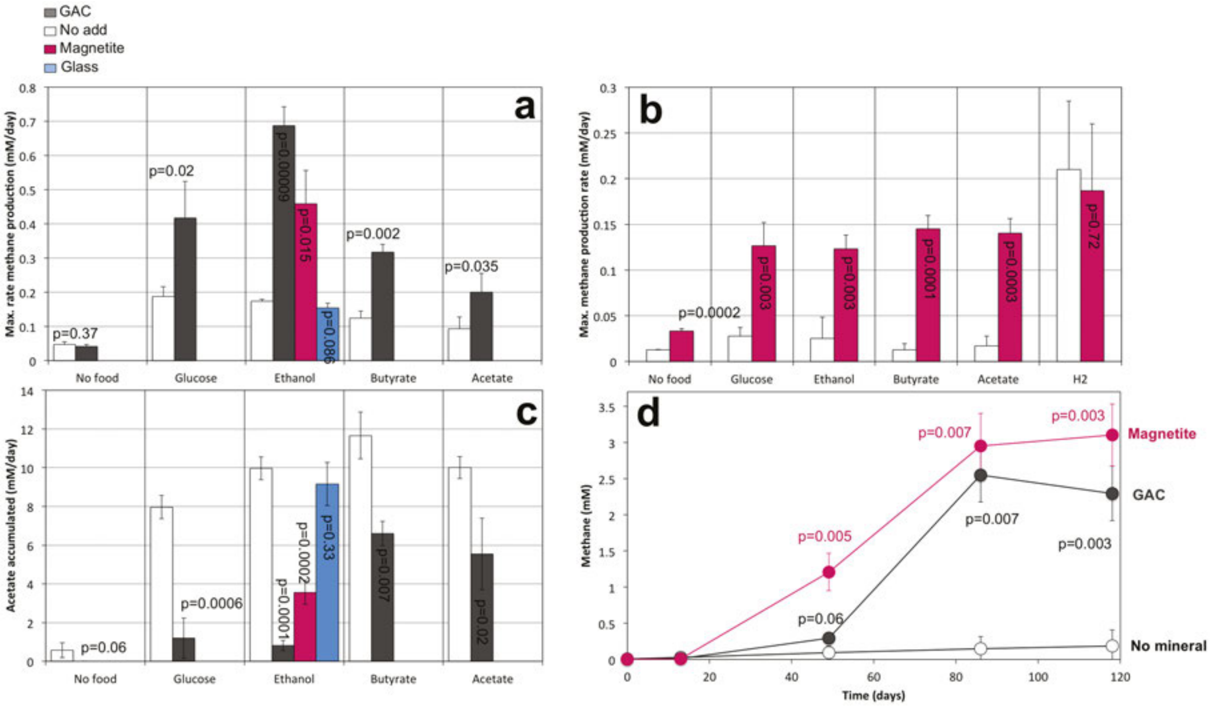

Figure 5. Methanogenesis on different substrates in incubations from lake La Cruz samples. Methane production in initial slurry incubations provided with different substrates was stimulated by conductive particles (GAC or magnetite) independent of the media used (a) modified DSMZ 120 or (b) DSMZ 334. (c) Acetate accumulated in incubations without conductive particles, but was significantly lower at the addition of conductive particles. (d) For example, a third transfer free of sediment showed that methanogenesis and acetate consumption were strictly dependent of the presence of conductive particles (colored symbols), and ceased if conductive particles were not added consistently for subsequent transfers (white symbols). Values represent average ± standard deviation and are based on triplicate incubations, with the exception of the incubation with acetate plus GAC, where we had duplicates. *Metanogénesis con diferentes sustratos en incubaciones con muestras del lago La Cruz. La producción de metano en las incubaciones iniciales de lodos provistos con diferentes sustratos fue estimulada por partículas conductivas (GAC o magnetita) independientemente del medio utilizado (a) DSMZ 120 modificado o (b) DSMZ 334. (c) Acetato acumulado en incubaciones sin partículas conductivas, que fue significativamente menor tras la adición de partículas conductivas. (d) Por ejemplo, una tercera transferencia libre de sedimento mostró que la metanogénesis y el consumo de acetato dependían estrictamente de la presencia de partículas conductivas (símbolos de color), y cesaban si las partículas conductivas no se añadían consistentemente para las transferencias subsiguientes (símbolos blancos). Los valores representan la media y la desviación estándar y están basados en incubaciones triplicadas, excepto la incubación con acetato incorporado de GAC, que fueron por duplicado.*

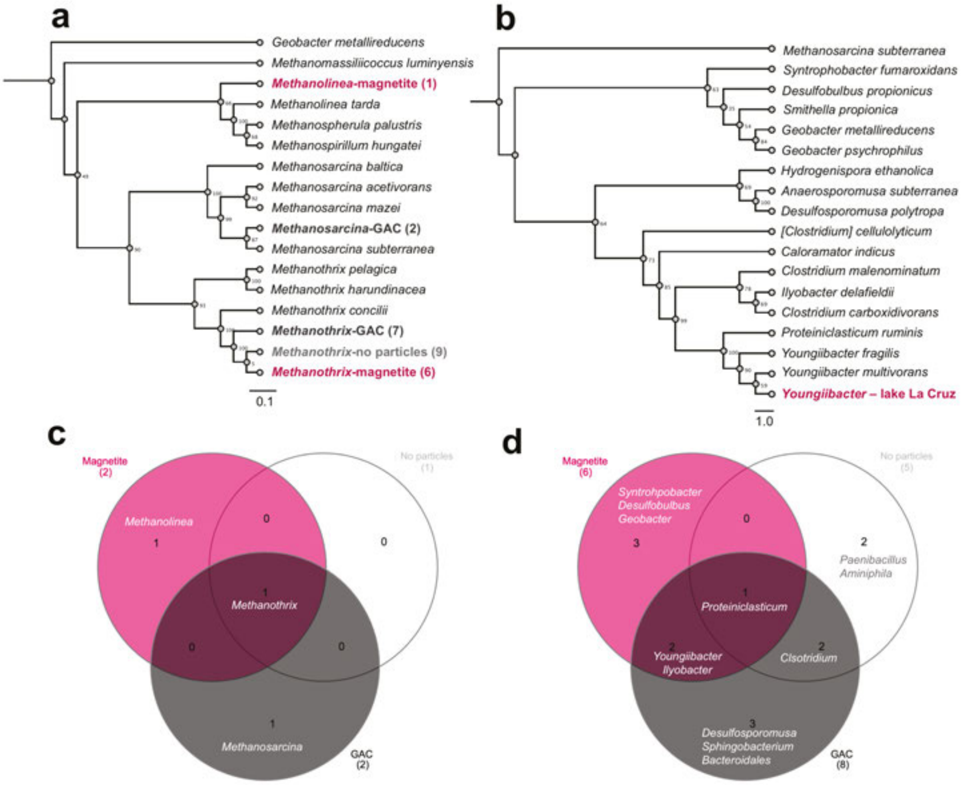

Figure 6. Maximum likelihood phylogenetic trees (a, b) and Venn diagrams with the relative distribution of 16S rRNA-gene sequences for Archaea (a, c) and Bacteria (b, d) in La Cruz incubations with or without conductive particles. Archaeal (a) and Bacterial (b) 16S rRNA genes were retrieved from third mud-free transfer of acetate-incubations with magnetite (pink), and GAC (black-bold) or from a first mud-free transfer without conductive particles (light gray-white). (c) The only Archaeal 16S phylotype encountered in all incubations independent of treatment was *Methanothrix*-related. (d) The most abundant Bacterial phylotype encountered only in conductive particle-amended incubations was *Youngiibacter*-related. *Árboles filogenéticos de máxima verosimilitud (a, b) y diagramas de Venn con la distribución relativa de las secuencias del gen 16S rRNA para Archaea (a, c) y Bacteria (b, d) en incubaciones de muestras de La Cruz con o sin partículas conductivas. Los genes Archaeal (a) y Bacterial (b) 16S rRNA se recuperaron de la tercera transferencia sin sedimento en incubaciones con acetato y magnetita (rosa) y GAC (negro fuerte) o de una primera transferencia sin sedimento y sin partículas conductoras (gris claro-blanco). (c) El único filotipo de 16S de Archaeas encontrado en todas las incubaciones, independiente del tratamiento, estaba relacionado con* Methanothrix. *(d) El filotipo bacteriano más abundante encontrado solo en incubaciones complementadas con adición de partículas conductivas fue del tipo relacionado con* Youngiibacter.

enriched on acetate with or without conductive particles. For this we compared the acetate fed communities exposed to two types of conductive particles (GAC and magnetite) to a community exposed to no conductive particles. We determined that *Youngiibacter* and *Methanothrix* methanogens dominated the enrichments amended with both types of conductive particle (Fig. 6). On the other hand, in controls without conductive particles, after only one single transfer *Youngiibacter* could not be detected. In the absence of conductive particles methane production only occurred slowly for one transfer and in this case *Methanothrix* co-existed with *Clostridium* (Fig. 6).

Youngiibacter was only found in enrichments with conductive particles and its presence could be associated with rapid acetate consumption coupled to methane production (Fig 5). We therefore anticipate that *Youngiibacter* plays a role in conductive-particle mediated syntrophy. Nevertheless, until now little is known about this group of Firmicutes, and only recently two species of *Youngiibacter* have been described (Lawson *et al.*, 2014; Tanaka *et al.*, 1991), of which one is associated with fermentation of organics on coal surfaces during coal conversion to natural gas (Lawson *et al.*, 2014). Coal, similar to activated carbon, is electrically conductive (Duba, 1977). Moreover, *Methanothrix* have been also found associated with coal conversion to natural gas (Beckmann *et al.*, 2011; Lawson *et al.*, 2014). It is therefore possible that *Youngiibacter* and *Methanothrix* play a role in conductive particle-mediated syntrophy in coal beds, and as well in Lake La Cruz sediments. However, a syntrophic association between *Youngiibacter* and *Methanothrix* has not been described before. We suggest that *Youngiibacter* released electrons from substrate/acetate oxidation onto conductive minerals that are then used as a source of electrons for *Methanothrix* in order to reduce CO_2 to methane. It is possible that *Youngiibacter* releases electrons extracellularly using a similar mechanism to that described for *Geobacter* namely a network of outer membrane *c*-type cytochromes (OMC) and pili (Shrestha *et al.*, 2013). During DIET, OMCs were not as necessary for a donor *Geobacter* strain to carry substrate oxidation coupled with extracellular electron transfer (EET) and respiration, since OMCs could be completely replaced by the conductive iron oxide, magnetite (Liu *et al.*,

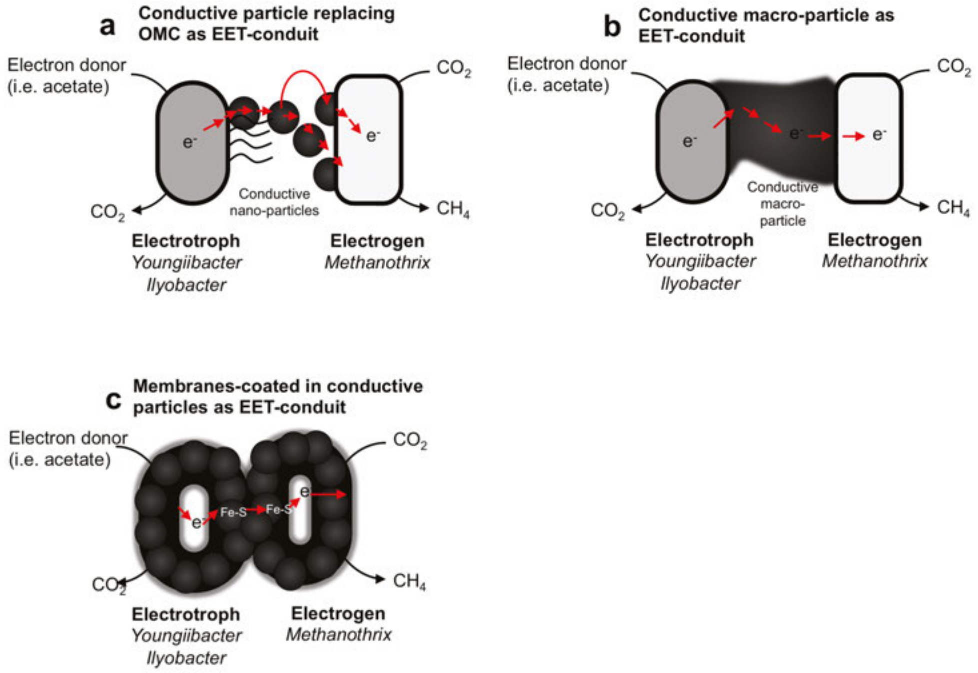

Figure 7. Proposed model interspecies interactions in La Cruz sediments facilitated by conductive particles. (a) Syntrophy mediated by a conductive nano-particles replacing outer membrane cytochromes (OMCs). Nevertheless, pili involved in EET are still available. (b) Syntrophy mediated by a conductive macro-particle (e.g. GAC), which plays the role of both and electron plug and outlet. (c) Syntrophy mediated by a conductive-mineral coat padding the cell surface. In lake La Cruz, conductive minerals could for example result from the precipitation of Fe^{2+} as Fe-S/thiol-. Cell surfaces encrusted with a Fe-S coat might endorse the electron-transfer between the two distinct metabolic entities even in the absence of a typical EET/DIET conduit. *Modelo de interacciones interespecies propuesto para los sedimentos de La Cruz facilitados mediante partículas conductivas. (a) Sintrofia mediada por nanopartículas conductivas que reemplazan a los citocromos de membrana externa (OMC). Sin embargo, los pili involucrados en EET todavía están disponibles. (b) Sintrofia mediada por una macropartícula conductiva (es decir, GAC), que desempeña el papel de ambos, la conexión y la fuente de electrones. (c) Sintrofia mediada por una envoltura mineral conductiva que rellena la superficie célular. En el lago La Cruz, los minerales conductivos podrían, por ejemplo, resultar de la precipitación de Fe*$^{2+}$ *como Fe-S/tiolatos- en el espacio periplásmico de las células. Las superficies celulares incrustadas con una envoltura de sulfuros metálicos como Fe-S podrían avalar la transferencia de electrones entre las dos entidades metabólicas distintas, incluso en ausencia de un típico conducto EET / DIET.*

2015). Instead, when it plays the role of electron donor *Geobacter* seems to necessitate e-pili for long range electron transfer to partner cells, as exemplified in a recent study (Ueki *et al.*, 2018). In agreement with previous observations in *Geobacter*, *Youngiibacter* might employ type IV pili for EET to partner *Methanothrix*. *Youngiibacter's* type IV pili gene sequence (T472_0202395) differs greatly from that of *Geobacter* in length (238 aminoacids longer than its correspondent in *G. metallireducens*) and sequence identity (only a stretch of 16 aminoacids showed 50 % identity). Nevertheless, this prepilin-gene encodes for a protein with high content of aromatic aminoacids (10.3 %) which could give this organism an advantage to carry EET (Walker *et al.*, 2018). It is possible that conductive particles ornate the pili of *Youngiibacter* in a similar way to how they do for *Geobacter* (Liu *et al.*, 2015; Wang *et al.*, 2018) facilitating electron transfer to syntrophic partner methanogens.

Conductive-particle mediated syntrophy

Syntrophy mediated by conductive particles could occur in three different ways (Fig. 7). A first mode of action includes electrogens with limited expression of surface cytochromes whose role would be replaced with that of conductive minerals (pyrite, pyrrhottite, magnetite, goethite) found in sediments (Fig. 7a). Molecular and microscopic evidence for this type of association has been brought by studies in laboratory *Geobacter* co-cultures provided with magnetite (Liu *et al.*, 2015). A second possibility is that cells plug into macro-sized conductive rocks (i.e. iron/manganese-nodules) with one cell releasing electrons onto the rock and the other receiving electrons (Fig. 7b). Evidence for such interactions was previously obtained in laboratory co-cultures with macro-sized conductive chars. In this case, using SEM, it was shown that the electrogen/*Geobacter* did not require direct contact to the electrotroph/*Methanosarcina* yet the conductive surface facilitated the syntrophic association (Chen *et al.*, 2014; Liu *et al.*, 2012). The third possibility (Fig. 7c), is that membrane-bound proteins facilitate the precipitation of Fe^{2+}-ions, e.g., with thiol groups (Milner-White & Russell, 2005) to form a conductive surface-conduit surrounding the cell. Extracellular electron transfer between such mineral-coated cells has been proposed (Kato *et al.*, 2012), but has not been confirmed. However, this could be a possibility for microbes without an extracellular apparatus for electron transfer to partner cells.

In evolutionary terms, it is plausible that mineral-mediated interactions preceded interspecies electron transfer interactions based in diffusible chemicals, which require complex enzymes and cell-bound electrical conduits. Primordial protocells had not developed enzymatic machineries to maintain redox and proton gradients across cell membranes (Martin *et al.*, 2003; Russell *et al.*, 1990, 1994; Wächtershäuser, 1988b). It has therefore been suggested that minerals, which can uphold voltage differences, such as FeS/pyrite, might have helped nucleate the earliest membranes, playing the role of early membrane-bound catalysts, instead of electron transport chain enzymes (Martin *et al.*, 2003). Later, the high reactive iron content of the Archaean ocean could have promoted the formation of proteins with Fe-S centers which are required and abundant in redox proteins of methanogens (Liu *et al.*, 2010). Here, we propose that primitive cells with leaky membranes (Lane & Martin, 2012), allowed easy electron transfer via conductive minerals permitting energy exchange between separate metabolic protocell entities. Thus, conductive particles could have fostered the earliest interspecies interactions in the methanogenic and iron-rich Early Earth oceans, and possibly nurtured adaptation of interspecies associations pre-eukaryogenesis.

CONCLUSION

In conclusion, we show that the sediment of an early Earth ocean analogue is a niche for syntrophic associations dependent on conductive particles. *Only if* conductive particles were provided, could syntrophic bacteria coupled to methanogens oxidize their substrates. Thus, only in incubations with conductive particles, members of the genus *Youngiibacter* co-existed with *Methanothrix*. Incubations without conductive particles resulted in the disappearance of *Youngiibacter*, and one transfer later to the demise of the methanogenic community. These data indicate that conductive particles were required to aid the pairing of the metabolism of *Youngiibacter* with that of *Methanothrix*, which sustained high rates of methanogenesis in this early Earth analogue – lake La Cruz. We propose that obligate mineral-syntrophy is an ancestral interspecies interaction established before complex membrane structures and enzymes evolved to mediate direct or indirect associations between species with distinct metabolism.

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The ‘unseen’ microbial diversity of a Spanish solution lake: ecological and other implications

Genoveva F. Esteban[1,*], Ken J. Clarke[2], Eduardo Vicente[3], Miracle M. R.[3] and Bland J. Finlay[4]

[1] Bournemouth University, Department of Life and Environmental Sciences, Talbot Campus, Poole, Dorset BH12 5BB, UK.

[2] Freshwater Biological Association, Windermere, Cumbria LA22 0LP, UK.

[3] Instituto Cavanilles de Biodiversidad y Biología Evolutiva, Universidad de Valencia, 46980 Paterna, Valencia (Spain).

[4] Queen Mary University of London, The River Laboratory, Wareham, Dorset BH20 6BB, UK.

[*] Corresponding author: gesteban@bournemouth.ac.uk

ABSTRACT

The ‘unseen’ microbial diversity of a Spanish solution lake: ecological and other implications

The ‘unseen’ microbial diversity of a small solution lake in central Spain is investigated using electron microscopy. The water has an elevated concentration of dissolved sulphate that supports significant abundance of sulphate reducing bacteria. Water samples were collected from the 13m stratum, fixed *in situ* and prepared for posterior electron microscopy examination. The results show high diversity of prokaryotes and microbial associations, particularly predatory bacteria, thriving in the anoxic layers of the water body. This high abundance and biodiversity, including multi-infestation of individual cells, increase the number of lateral links in the otherwise impoverished anaerobic food chain. The wider potential applications of predatory bacteria are discussed.

Key words: predatory bacteria, sulphide, anaerobic ciliates, *Chromatium*, carbon cycls

RESUMEN

Biodiversidad microbiana ‘oculta’ en un lago cárstico

La diversidad microbiana "oculta" de un pequeño lago cárstico situado en el centro de España se investiga mediante microscopía electrónica. El agua tiene una concentración elevada de sulfato disuelto que sostiene una alta abundancia de bacterias reductoras de sulfato. Se recogieron muestras de agua del estrato situado a 13 m de profundidad, las cuales se fijaron ‘in situ’ *y se prepararon para su posterior observación con microscopía electrónica. Los resultados muestran una gran diversidad de procariotas y de asociaciones microbianas, particularmente de bacterias depredadoras, que prosperan en las capas anóxicas del cuerpo de agua. Esta gran abundancia y biodiversidad, incluida la infestación múltiple por bacterias depredadoras, tiene como consecuencia el aumento del número de enlaces colaterales de la cadena alimentaria anaeróbica, que de otro modo es una cadena muy empobrecida. Estas observaciones permiten deliberar sobre las posibles aplicaciones de bacterias depredadoras.*

Palabras clave: *bacterias depredadoras, sulfato, ciliados anaerobios,* Chromatium*, ciclo del carbono*

INTRODUCTION

Our collaborative research over the years mostly focussed on aquatic microbial eukaryotes, particularly the biodiversity of anaerobic ciliates in solution lakes in central Spain. These investigations yielded the discovery of new ciliate species (Esteban *et al.*, 1993), first accounts of ciliates' associations with endosymbiotic (methanogenic and other) archaea for these habitats (Finlay *et al.*, 1991), and the description of difficult-to-document prokaryotic life cycles (Clarke *et al.*, 1993). Despite this wealth of discoveries there still remained a significant amount of microbiological material collected during the intense sampling summer campaigns that, although it was examined, it was never published, and it remained 'unseen'. This is precisely the aim of this *'in memoriam'* article – to bring to light some of the interesting prokaryote associations, some (probably) predator/prey related, which were observed and documented from these remarkable anoxic aquatic habitats. We also discuss the ecological and other potential implications of these microbial consortia.

MATERIAL AND METHODS

Study site, sampling and electron microscopy

Samples were collected within the 13-metre stratum in Arcas-2, a small solution lake (formed by dissolution of gypsum-rich marls) in Cuenca, central Spain. The lake has a relatively constant depth of 14 m, with the bottom 7 m being anoxic during the summer months (Finlay *et al.*, 1991). The water has an elevated concentration of dissolved sulphate that supports significant bacterial sulphate reduction (Camacho *et al.*, 2000; Rodrigo *et al.*, 2000). Water samples from within the 13 m deep anoxic layer (approximately 4.5 m below the oxic-anoxic boundary) were collected using a pump sampler; samples were kept in an air-free, air-tight bottle in the dark until their immediate processing (see below). Detailed sampling methods have been described previously (Finlay *et al.*, 1991). Prof. Miracle was involved in the sample collection for recording data, and also in the *'in situ'* examination of the living and fixed samples. On the banks of Arcas-2, each sample was immediately given a range of preparation procedures for Electron Microscopy, based on the standard fixation of glutaraldehyde followed by osmium tetroxide (Clarke *et al.*, 1993). Back at the Ferry House on the Windermere shores (England, UK) the embedded and cut sections were stained with our usual triple stain lead citrate/uranyl acetate/lead citrate, before examination with Transmission Electron Microscopy. All the samples were collected in 1992, and photographed in early and mid-1993.

RESULTS AND DISCUSSION

High abundance and biodiversity of prokaryote 'morphotypes' were observed in both, the environmental water samples and inside food vacuoles of anaerobic ciliates. Figure 1 shows a snap-shot of the morphological diversity of prokaryotes in one single sample and one single Electron-Microscop-ultra-thin section photograph: 140 cells counted belonging to 24 morphotypes. One of the most common prokaryotes observed is a 'winged' bacterium found in the water and in food vacuoles of ciliates inhabiting the anoxic layers of Arcas-2 (Fig. 2; see also Finlay *et al.*, 1991), demonstrating the active role of anaerobic ciliates in predating upon bacteria, and their contribution to the carbon-sulphur cycles in this lake. The 'winged' appearance of the bacteria shown in Figs. 1 and 2 corresponds to filaments of overlapping cells into one another – hence the 'wings'. These short bacterial filaments are probably sulphate reducers, and the inclusions seen inside them (Figs 1, 2) are probably either sulphur or gas (Finlay *et al.*, 1991).

The high productivity of Arcas-2 is stimulated by the significant supply of sulphate (from ground water percolating through gypsum), which sustains the growth of sulphate-reducing bacteria in the anoxic layers of the lake (Clarke *et al.*, 1993; Vicente *et al.*, 1991). The sulphide diffuses to upper layers of the water column and up to the metalimnion, being consumed along its way by phototrophic bacteria like *Chromatium*, providing an interesting link between the carbon and sulphur cycles (Finlay *et al.*, 1991) in the anoxic worlds. *Chromatium* is a significant

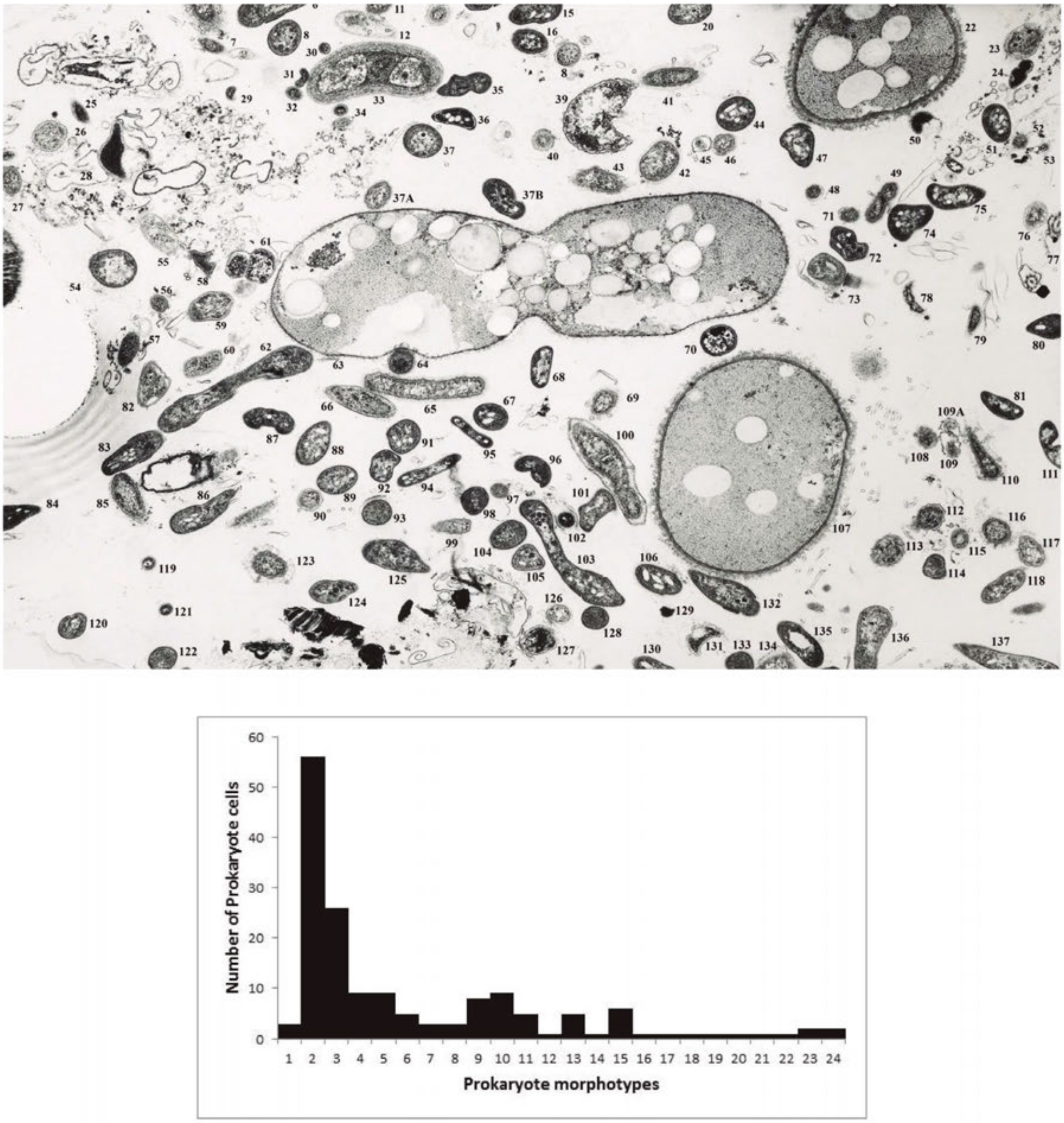

Figure 1. Transmission Electron micrograph (top) showing diversity of prokaryote morphotypes in one single ultrathin-section from one single sample from 13 depth in Arcas-2 (Spain). Total number of cells: 140, belonging to 24 different morphotypes. The ‘winged’ bacteria is one of the most abundant prokaryotes, see, e.g. 6, 14, 15, 93, 103, 132; note also the abundance of prokaryote associations, e.g. 31 with 33, 63 with 64, 100 with 101, 103 with 128. The black rectangle at the top right of the image corresponds to the TEM film negative number. Bottom image shows the individual abundance of each of the 24 morphotypes. Scale bar = 1 µm. *Micrografía electrónica de transmisión (imagen superior) mostrando la diversidad de morfotipos procariotas en una sola sección ultrafina de una sola muestra de 13m de profundidad en Arcas-2 (España). Número total de células: 140, pertenecientes a 24 morfotipos diferentes. La bacteria "alada" es uno de los procariotas más abundantes, ver, p. 6, 14, 15, 93, 103, 132; nótese también la abundancia de asociaciones procariotas, p. 31 con 33, 63 con 64, 100 con 101, 103 con 128. El rectángulo negro en la parte superior derecha de la imagen corresponde al número de negativo de la película TEM. La imagen inferior muestra la abundancia individual de cada uno de los 24 morfotipos. Barra de escala = 1 µm.*

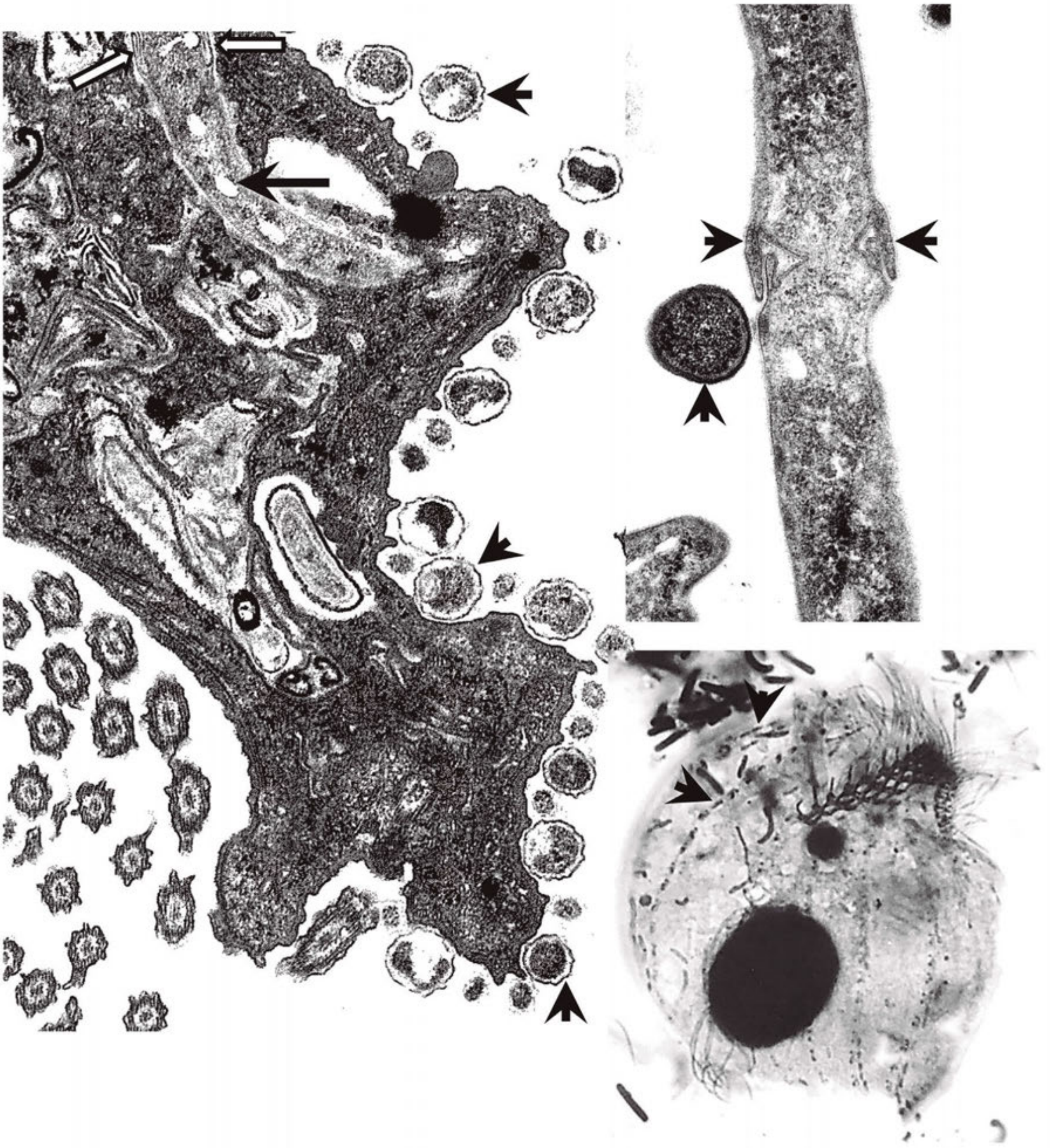

Figure 2. Transmission Electron micrographs of a ciliate (left, probably *Caenomorpha* sp.) showing the ectosymbiotic bacteria (arrows) on the ciliate surface, and the 'winged' bacteria inside the food vacuole (white arrows, top of image). The 'winged' bacterium (right) were also observed bearing prokaryote attached to their surface. The bottom image shows the ectosymbiotic bacteria aligned on the surface of the ciliate *Saprodinium difficile*, scale bar for this image = 5µm. *Micrografías electrónicas de transmisión de un ciliado anaerobio (izquierda, probablemente* Caenomorpha *sp.) que muestra las bacterias ectosimbióticas (flechas) en la superficie del ciliado y las bacterias "aladas" dentro de la vacuola alimentaria (flechas blancas, parte superior de la imagen). La bacteria "alada" (derecha) también se observó con procariotas unidos a su superficie. Barras de escala = 0.5 µm. La imagen inferior muestra una micrografía (microscopía óptica) de las bacterias ectosimbióticas alineadas en la superficie del ciliado anaerobio* Saprodinium difficile*; barra de escala para esta imagen = 5 µm.*

Figure 3. Transmission Electron micrographs showing associations between prokaryotes from 13m depth in Arcas-2, Spain. Some bacteria were associated with more than one prokaryote, probably predatory bacteria. Scale bars = 0.5µm. *Micrografías electrónicas de transmisión que muestran asociaciones entre procariotas encontradas a 13 m de profundidad en Arcas-2, España. Algunas bacterias se asociaron con más de un procariota, y son probablemente bacterias depredadoras. Barras de escala = 0.5 µm.*

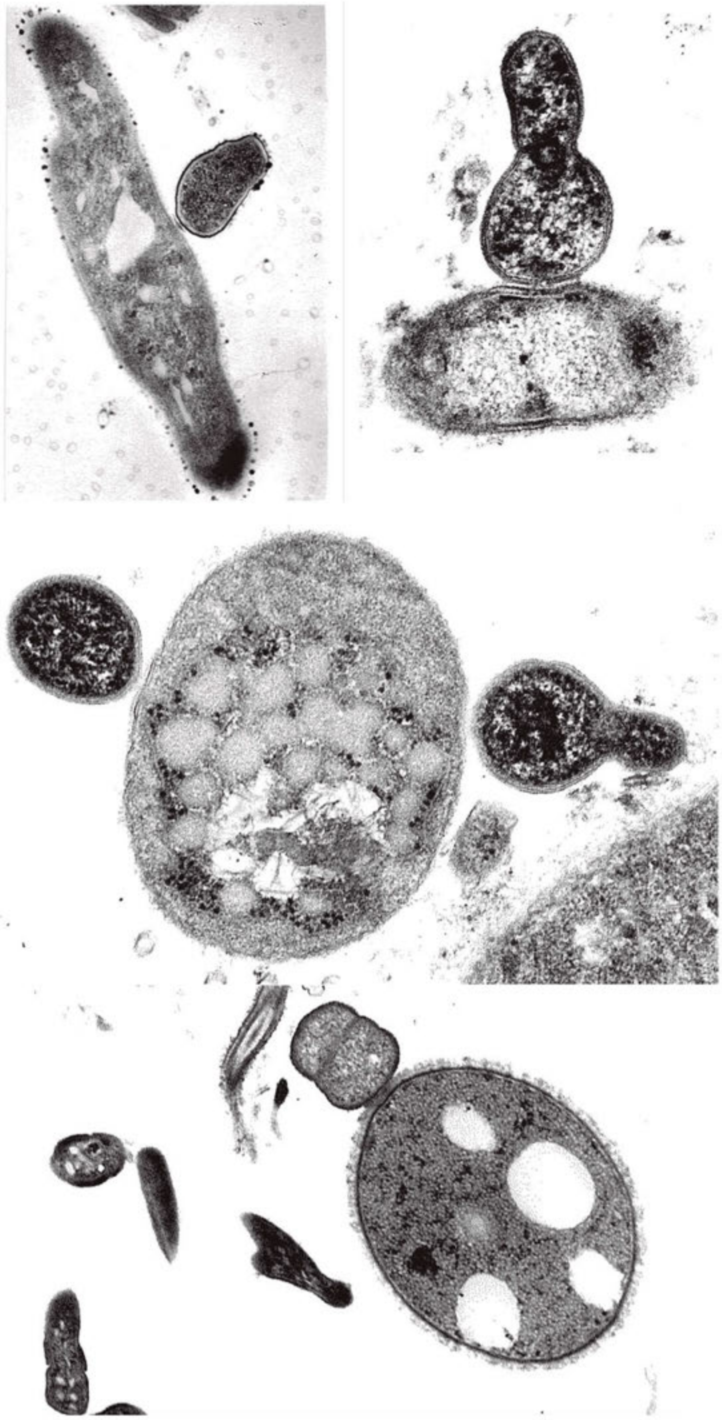

Figure 4. Transmission Electron micrographs of further microbial associations found at 13m depth in Arcas-2 (Spain). Some bacteria were associated with more than one prokaryote, probably predatory bacteria. Bottom image shows the prokaryote epibiont of the photosynthetic bacterium *Chromatium weissei*. Scale bars = 0.5µm. *Micrografías electrónicas de transmisión de otras asociaciones microbianas encontradas a 13 m de profundidad en Arcas-2 (España). Algunas bacterias se asociaron con más de un procariota, probablemente bacterias depredadoras. La imagen inferior muestra el epibionte procariota de la bacteria fotosintética* Chromatium weissei. *Barras de escala = 0.5 µm.*

source of food for ciliates in these environments (Finlay *et al.*, 1991); carbon fixed by these phototrophic bacteria that are ingested and subsequently digested by anaerobic ciliates is incorporated into ciliate biomass. Furthermore, since most anaerobic ciliates in Arcas-2 harbour endosymbiotic methanogenic archaea (Finlay *et al.*, 1991) that require hydrogen for their growth, the carbon fixed from the digested phototrophic bacteria will also be used in archaeal methane production that is eventually released into the surrounding water and then into the atmosphere. The sulphur ingested by the ciliates will probably be eliminated via the usual cytoproct's waste removal process.

The external surface of some of the anaerobic ciliates found in Arcas-2 has rod-shaped ectosymbiotic prokaryotes attached to it (Fig 2; Esteban *et al.*, 1993), adding another layer of complexity to the microbial food web found in the lake's anoxic water. Further examples of microbial consortia in this habitat are delivered by the high abundance of other types of prokaryote associations; in some instances, bacteria have two or more prokaryotes fastened to their surfaces (Fig. 1), like *Chromatium* (Fig. 4); others seem to be predators (Figs. 2, 3), with some bacteria being simultaneously infested by several bacterial predators as well as epibionts (Fig. 3).

Anaerobic food chains are very short due to the much lower energy yield of anaerobic metabolism (Fenchel & Finlay, 1995), where protozoa are the only top predators able to complete the life cycle in total absence of oxygen. The string of diverse microbial associations found in the anoxic water of Arcas-2 increases the number of lateral links in these short anaerobic food chains, e.g. the heterotrophic prokaryote on the surface of the phototrophic *Chromatium* (Fig. 4) probably depends exclusively on the DOC produced and excreted by the latter (Clarke *et al.*, 1993), and the predatory bacteria in anoxic water contribute to the 'recycling' of carbon.

Predatory prokaryotes have long been known in the scientific literature, e.g. *Vampirococcus*, *Bdelovibrium*, *Daptobacter* (Guerrero *et al.*, 1986). This predatory behaviour provides a window into early evolution of eukaryotes, showing how bacteria-bacteria predation can enlighten the origin of intracellular organelles (see e.g. Fig 3, and Guerrero *et al.*, 1986, 1987; Jurkevitch & Davidov, 2007). Furthermore, research on 'predatory bacteria' is currently a promising line of enquiry into the fight against antibiotic-resistant bacteria. It has successfully been experimented with the predatory *Bdellovibrium* and *Micavibrio* – both bacteria have the ability to prey and reduce many multidrug-resistant pathogens associated with human infection (Dashiff *et al.*, 2011). The use of predatory bacteria (also known as 'living antibiotics') has been proposed as potential solution to the rise of multidrug-resistant bacterial infections (Kadouri *et al.*, 2013; Shatzkes *et al.*, 2016; Dwidar & Yokobayashi, 2017; Madhusoodanan, 2019).

The 'unseen' microbial diversity of Arcas-2 has allowed us to reveal a variety of prokaryotes and microbial associations that thrive in the absence of oxygen in sulphate-rich solution lakes, their ecological importance in increasing the number of trophic interactions in the low energy-yield short anaerobic food chains, and how bacteria-bacteria predation may hold the key to a post-antibiotic future. Most importantly, it reveals how the intricacies and potentials of microbes remain largely unknown.

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Drivers of the *Ceratium hirundinella* and *Microcystis aeruginosa* coexistence in a drinking water reservoir

István Grigorszky[1,2,*], Keve Tihamér Kiss[2], László József Szabó[1], György Dévai[1], Sándor Alex Nagy[1], Imre Somlyai[1], Csaba Berta[1], Marija Gligora-Udovič[3], Gábor Borics[2], Gabriella Pór[4], Yaqoob Majd Muwafaq[1], Arber Hajredini[1], Uyanga Tumurtogoo[1] and Éva Ács[2]

[1] University of Debrecen, Department of Hydrobiology, H-4032 Debrecen, Egyetem tér 1., Hungary.
[2] Hungarian Academy of Sciences, Centre for Ecological Research, Danube Research Institute, H-1113 Budapest, Karolina út 29-31., Hungary.
[3] University of Zagreb, Faculty of Science, Department of Biology Rooseveltov trg 6, 10000 Zagreb, Croatia.
[4] Paterson Johal Consulting, #203B – 10190 152A Street, Surrey, BC, V3A 1M8 Canada.

[*] Corresponding author: grigorszky.istvan@science.unideb.hu

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ABSTRACT

Drivers of the *Ceratium hirundinella* and *Microcystis aeruginosa* coexistence in a drinking water reservoir

The spatial and temporal patterns of phytoplankton in relation to physical and chemical properties were studied in a drinking water reservoir - the Lázbérc Reservoir - located in north-east Hungary. The main objectives were to determine the role of the hydrologic regime in structuring algal growth and the effects of physical and chemical variables on the coexistence of a dominant species: cyanobacteria, *Microcystis aeruginosa* (Kützing) Kützing and an eukaryotic dinoflagellates, *Ceratium hirundinella* (O.F. Müller) Dujardin. The results suggest that nutrients (TP, NH_4–N and NO_3–N), turbidity, and the hydrologic regime play an important role in regulating the occurrence of investigated species. The rainy summer period resulting in a mixing of the water column, and the low level of nitrogen coupled with the internal release of phosphorus from the lake sediment under brief periods of anoxia promote cyanobacterial bloom.

During this period, the water column was characterised by high turbidity, which created favourable conditions for developing a huge *C. hirundinella* biomass and the simultaneous occurrence of *M. aeruginosa*. This study also demonstrated - that contrary to what was previously known, deep standing waters in the temperate zone are not only dimictic (mixing twice a year during the spring and the fall), but they can also be mixed during the summer period due to continued rainfall, which equalizes the temperature in the whole water column. This can promote the occurrence of cyanobacterial and eukaryote planktonic organisms.

Key words: *Microcystis aeruginosa*, *Ceratium hirundinella*, phosphorus, seasonal variation, water management, lake-polymixis in temperate zone

RESUMO

Efeito da flor de* Ceratium hirundinella *e* Microcystis aeruginosa *no reservatório de água potável

Os padrões espaciais e temporais do fitoplâncton e sua correlação com as especificidades variáveis físicas e químicas, foram examinados no nordeste da Hungria, localizados em um reservatório típico de água potável no reservatório de Lázbérc. Nosso principal objetivo foi para esclarecer o papel da hidrologia, e os diferentes especificidades físicas e químicas na coexistência de espécies dominantes: cianobactéria, Microcystis aeruginosa *(Kützing) Kützing e* Dinoflagellata eucariótica, Ceratium hirudinella *(OF Müller) Dujardin. De acordo com os resultados, os nutrientes (TP, NH_4-N, NO_3-N), turbidez e os condições hidrológicas desempenham um papel importante na regulação da ocorrência das espécies examinadas. O período de verão chuvoso que resulta um equilíbrio do nível da água, o baixo nível de nitrogênio, juntamente com a liberação interna de fósforo do sedimento, durante um curto período anóxico promove a floração de cianobactérias. Durante este período, a coluna de água foi caracterizada por alta turbidez, criando condições favoráveis para o surgimento de uma enorme expansão da biomas-*

sa C. hirundinella *e a aparição simultânea de* M. aeruginosa. *Este estudo mostrou também que as águas profundas nas áreas da zóna moderada, não tem apenas dimictic especialidade (misturando duas vezes por ano, na primavera e no outono), mas também durante a estação do verão pode ser misturada por chuvas contínuas que compensam a temperatura em toda a coluna da água. Isso ajuda a presença de cianobactérias e organismos eucariotas planctônicos.*

Palavras chave: Microcystis aeruginosa*,* Ceratium hirundinella*, fósforo, variação sazonal, manejo da água, lago-polimixis em zona temperada*

INTRODUCTION

Over recent years it has become clear that eutrophication is a significant problem in many freshwater reservoirs used as drinking water supply. Eutrophication includes high levels of chlorophyll-*a* (Boynton *et al.*, 1982; Nixon & Pilson, 1983; Conley & Malone, 1992), excessive macrophyte biomass and algal blooms, the occurrence of anoxia and hypoxia (Gerlach, 1990), and harmful and toxic algal blooms (Franks, 1997; Seda *et al.*, 2000; Oudra *et al.*, 2002; Jeppesen *et al.*, 2015).

The understanding of the factors behind the abundance patterns of microbial species in nature are a central issue in environmental sciences. Planktonic microorganisms, as the primary producers, can mirror changes in aquatic ecosystems and carry the basic information needed to assess the trophic status and water quality of aquatic environments. In freshwater phytoplankton assemblages, it is often observed that the phytoplankton undergoes a series of seasonal changes in individuum numbers and biomass, as well as shifts in composition. Succession has acquired a wide meaning in plankton ecology, covering the pattern of annual development, and the sequential changes in species dominance (Smayda, 1980). Planktonic microorganism development occurs at different temporal scales: eg. short-term variations induced by local weather are driven by stochastic disturbances, while annual variations lead to seasonal succession. Their development is the outcome of the interplay between both internal community-directed processes and external forcing (Kiss, 1996; Reynolds, 2000; Reynolds, 2003), and at the same time, abiotic and biotic factors. Nutrient enrichment often leads to increases in microbial biomass (Jones & Knowlton, 1993), and in particular cyanobacterial production and dominance (van Liere & Walsby, 1982; Izaguirre, 1992; Izaguirre *et al.*, 1998, 2007; Bouvy *et al.*, 2003; Izydorczyk *et al.*, 2009; Noyma *et al.*, 2016).

C. hirundinella is also widespread in lakes and ponds in temperate regions. it is often dominates the warm water period and at times also results in blooms also. (Reynolds 1976, Heaney 1976), However there has been little investigation on its population dynamics in reservoirs. Based on a literature review, *C. hirundinella* is a ubiquitous, relatively large and slow-growing species characteristically found during late summer in reservoirs, and in water bodies with a warm stable epilimnion and low nutrient concentrations (Nicolls *et al.*, 1980; Whittington *et al.*, 2000; Inkel *et al.*, 2001; Pérez-Martinez & Sánchez-Castillo, 2001, 2002; Grigorszky *et al.*, 2003). Under these low-turbulence conditions, its ability to undertake significant diel vertical migration enables an optimal exploitation of light and nutrients – two essential resources – whose availability contrasts directly in the vertical dimension during stratification (Heaney, 1976; Heaney & Furnass, 1980; James *et al.*, 1992; Whittington *et al.*, 2000). The seasonal periodicity of *C. hirundinella* is variable. Pérez-Martínez and Sánchez-Castillo (2001) reported clear winter maxima for an array of Spanish reservoirs, but subsequently further highlighted the variability of its occurrence, as reported in the literature. Notwithstanding its putative advantage at low nutrient levels (Whittington *et al.*, 2000), *C. hirundinella* also occurs in eutrophic lakes (e.g. Frempog, 1984), and was typified as a component of late-summer phytoplankton associations in oligotrophic, mesotrophic and eutrophic temperate waters (Reynolds, 1996). In South Africa, Ceratium blooms have been recorded in 17 of 57 reservoirs (van Ginkel *et al.*, 2007). Among these reservoirs, blooms occurred in all seasons and across the trophic status range. These studies show that *C. hirundinella* is a common organism in different seasons and in different trophic statuses, but no precise explanation is yet available for its occurrence. Furthermore, although the two species *M. aeruginosa* and *C. hirundinella* are frequently found together, accurate explanations for their co-occurrence are still lacking. However, the turbid conditions created by the heavy rain and the wind disturbance may have been more favorable for cyanobacterial growth since some taxa have a greater tolerance for turbid conditions (Kosten *et al.*, 2011). In addition, there may have been a positive feedback in the reservoirs with the increased biovolume of cyanobacteria caused by diel vertical migration promoting more turbid conditions, which in turn, enhanced cyanobacterial growth (Kosten *et al.*, 2011). Presing (1996) found that low light conditions created by cyanobacterial blooms facilitated their persistence. Nuisance cyanobacterial blooms are common in drinking water reservoirs and have been associated with the occurrence of objectionable taste and odor events (Saadoun 1999; Smith *et al.*, 2002). *Microcystis aeruginosa* is one of the dominant species of cyanobacteria that form surface water blooms in eutrophic lakes and has received much attention as a result of the water management problems associated with its blooms (Dokulil & Teubner 2000). The distribution and abundance of *M. aeruginosa* are to a large extent affected by factors such as light and nutrient availability acting synergistically with other physical and chemical factors. Nitrogen and phosphorus are generally considered to be the main nutrients for algal growth in temperate standing waters. It has been suggested that the lower ratio between these two nutrients may promote the development of cyanobacterial blooms (Smith, 1983). There is also an interesting theory, accompanied by some investigations that positively buoyant cyanobacteria, such as *Microcystis*, have an ecological advantage in light-limited waters due to buoyancy regulation (Huisman *et al.*, 2004;). Therefore it is thought that low light and low nitrate concentrations may create conditions more favourable to the dominance of *M. aeruginosa*. However, little information is available about the combined effects of nitrogen, phosphorus concentration, and light intensity on population dynamics and coexistence with other population of *M. aeruginosa*. We conducted a field experiment to evaluate the nutrient concentrations and the hydrological regime in combination with light intensities on the growth/coexistence of *C. hirundinella* and *M. aeruginosa* in a drinking water reservoir. The objectives of this study were to determine (i) temporal and spatial patterns in the relationships between water quality and cyanobacterial and dinoflagellates growth, (ii) the role of the hydrologic regime in structuring cyanobacterial growth and (iii) which variables make it possible for the two dominant taxa, *M. aeruginosa* (cyanobacteria) and *C. hirundinella* (eukaryotic alga, dinoflagellate), to coexist. In addition, we discuss the implication of our results with respect to management strategies for the Lázbérci Reservoir and drinking water reservoirs in general.

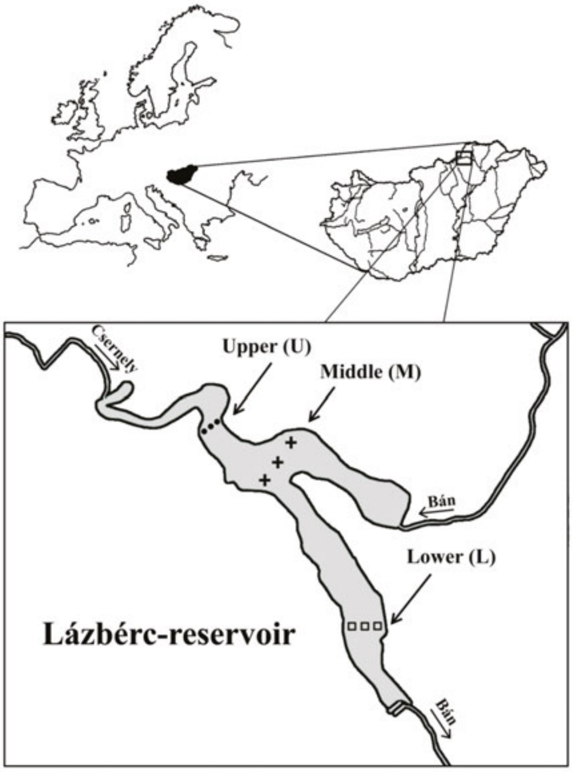

Figure 1. Sampling sites at the studied Lázbérci Reservoir. *Locais de amostragem no reservatório de Lázbérc.*

MATERIAL AND METHODS

Lázbérci Reservoir is located in the North-Eastern part of Hungary, in a low mountain region (48.1226 ° N, 20.2752 °E, Fig. 1). It was constructed in period between 1967 and 1969 to assure the drinking water supply for the fast-growing region of North-Hungary. The reservoir volume is 5.9 million m^3. Its average depth is 7.5 m and its maximum depth is 17.2 m. The reservoir has a surface area of 77 ha that can be extended to 92.2 ha during flood control operations. The 23.9 km long Bán Stream and the 17.2 km long Csernely Stream flow into the reservoir. The retention time of the reservoir is approximately 1 year. The watershed encompasses nearly 217.5 ha. Annual precipitation in the reservoir area was 806 mm in 2007 and 911 mm in 2008. The average annual precipitation in Hungary is 500-700 mm. The temperature reached its maximum value in July, in both years. By contrast, wind intensity increased in spring (April–June) and late summer to autumn (September to November). Reservoirs exhibit distinct longitudinal gradients in their physical, chemical, and biological properties due to their "river-lake hybrid" nature. In order to characterize these gradients, nine sampling sites based on lake morphology (i.e., location and depth) were selected and divided into three general categories (upper, middle and lower regions, Fig.1).

Each of the nine sampling sites was sampled monthly from April to November. A 2 L Van-Dorn sampler was used to collect water samples at a series of discrete depths that were determined by the maximum depth at each site (surface/0.25 m, 1.5 m, 3 m, 6 m, 9 m and 12 m). Phytoplankton counting was done according to Utermöhl (1931) in an inverted microscope (Axiovert-100). At least 400 units were counted giving a counting accuracy, expressed in terms of 95 % confidence limits of, < 10 % for the whole phytoplankton. The phytoplankton biomass determination was based on the calculation of the species volume.

Samples for NO_3–N, NO_2–N, NH_4–N, and PO_4–P were filtered through ion chromatography acrodisc 0.45 µm filters before analysis. Temperature, conductivity, oxygen, pH were measured when sampling. Total nitrogen (TN) and total phosphorus (TP) concentrations were determined using photometric procedures (Ebina *et al.*, 1983). The total alkalinity was measured titrimetrically (APHA, 1995). All laboratory analysis was performed within 48 hours of sample collection. The concentration of chlorophyll-*a*, corrected for pheophytin-a, was determined photometrically.

The Shapiro-Wilks test was used to analyze the normality of conductivity, TP and NH_4 within the groups. Multivariate normality conditions for the multivariate Canonical Variate Analysis (CVA) were analysed by the Mardin test for conductivity, TP and NH4. Since the latter test gave very similar results to the normal distribution, the upper, middle and lower parts of the reservoir were compared by CVA. The groups were compared with one-way ANOVA and Tukey post hoc (normal distribution) tests, or with the Kruskall-Wallis and Mann-Withney tests (non-normal distribution).

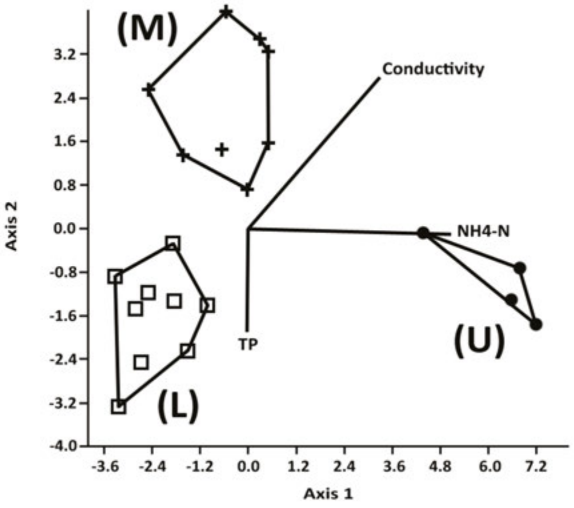

Figure 2. Canonical Variate Analysis (CVA) of the sampling sites in correspondence with environmental variables in Lázbérc Reservoir Canonical Variate 1 was significant (p < 0.0001) and comprised 74.76 % of the total variation. Canonical Variate 2 was also significant (*p* < 0.0001) and comprised 25.24 % of the total variation. (• = no species; + = *C. hirundinella* only; □ = *M. aeruginosa* and *C. hirundinella*). *Análise canônica multivariada (AVC) dos locais de amostragem em ligação de variáveis ambientais do reservatório Lázbérc. A variação canônica 1 foi significativa (p < 0.0001) e 74.76 % da variação total. A variação canônica 2 também foi significativa (*p *< 0.0001) e continha 25.24 % da variação total. (• = Nenhuma espécie; + = apenas* C. hirundinella*;* □ = M. aeruginosa *e* C. hirundinella*)*.

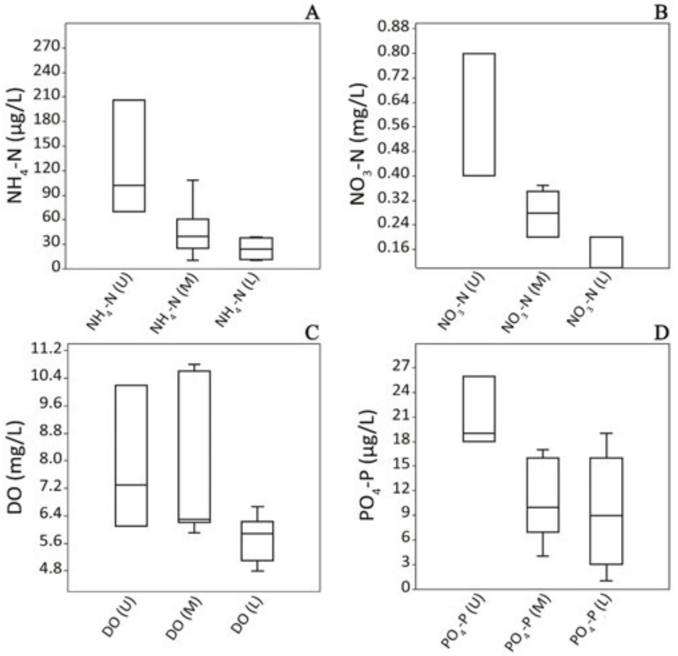

Figure 3. The physical and chemical variables in the various parts of the reservoir (U = upper region, M = middle region, L = lower region): A, NH_4-N; B, NO_3-N; C, dissolved oxygen; D, PO_4-P. *Especialidades físicas e químicas estudadas em três regiões do reservatório (U = região superior, M = região média, L = região inferior): A,* NH_4*; B,* $N0_3$*-N; C oxigénio dissolvido; D,* PO_4*-P.*

RESULTS

The CVA of the biomass of *M. aeruginosa* and *C. hirundinella* as regards to Total Phosphorous (TP), ammonium-ion (NH_4-N) and conductivity showed that the studied sites of the reservoir could be divided into three different groups (Fig. 2). Sampling sites, – the upper part of the reservoir, U – with high concentrations of ammonium-ion correlated with sites where neither *M. aeruginosa* nor *C. hirundinella* was present. The second group of sampling sites – middle part of the reservoir, M – was characterized by high conductivity and the presence of *C. hirundinella* only. The third group contained locations – the lower part of the reservoir, L –, where both species were present, and where the principle of separation was based on the low phosphorous content.

Based on the average concentrations of ammonium-ion, nitrate-ion and orthophosphate-ion the sampling sites were split into two groups (Fig. 3). The first group was composed of upper region samples with no presence of *C. hirundinella* or *M. aeruginosa* and with high concentration of the ammonium-ion, nitrate-ion and orthophosphate-ion. The second group represented sites from the middle and lower regions where *C. hirundinella* occurred on its own or sites where both species were found. In the second group the ammonium-ion, nitrite ion and orthophosphate-ion concentrations where low (Fig. 3),

Based on the average concentrations of dissolved oxygen, the sampling sites were split into two groups (Fig. 3C). Higher dissolved oxygen concentrations characterized sites where neither of the two species occurred or sites where *C. hirundinella* occurred on its own. Lower dissolved oxygen concentrations were measured at sites where both species were found.

Furthermore, we examined the variables that were influential in terms of the co-occurrence of the two species in the lower part of the reservoir, especially concerning the low dissolved oxygen concentrations. In May, dissolved oxygen levels were low (0-3 mg/L) in the lower regions (below 9 m depth) of the reservoir (Fig. 4A). In June and

July, the low oxygen content became characteristic of shallower depths, up to 1.5 m. By September, this low oxygen zone descended again below 3 m, and continued to descend below 9 m in October. In November, the dissolved oxygen content of the entire water column was significantly higher. In May and June, the orthophosphate-ion concentration was typically low both close to the surface and in deeper water layers (Fig. 4B). The observed ortophosphate-ion concentration increase in June and July was almost characteristic for the entire water column (Fig. 4B). In September, the orthophospate deficient water layer was sank down to a depth of about 4 meters (Fig. 4B), while this layer was characterized by a mass occurrence of the examined species (Fig. 4. C, D). In April and May, *C. hirundinella* did not occur at any depth (Fig. 4C). In June, there was a minor occurrence of the species at the upper two sampling depths, while it disappeared again in July. In September, the *C. hirundinella* biomass became significant in all three upper sampling depths, reaching the maximum value of the study period at 1.5 m (1.6 mg/L). In October, *C. hirundinella* only occurred at the upper two sampling depths with a low biomass.

In April and May, *M. aeruginosa* did not occur at any depth (Fig. 4D). In June, the species occurred at the 3 m depth in moderate numbers, which remained the same in July. In September, the biomass of *M. aeruginosa* increased significantly at all depths down to 3 m, reaching the maximum value (0.8 mg/L) at 1.5 m. The species did not occur in October and November.

In April, water temperature was at its minimum. All layers of water reached their maximum values in July. By this time, the temperature of the near-surface and the 1.5 m layer was higher

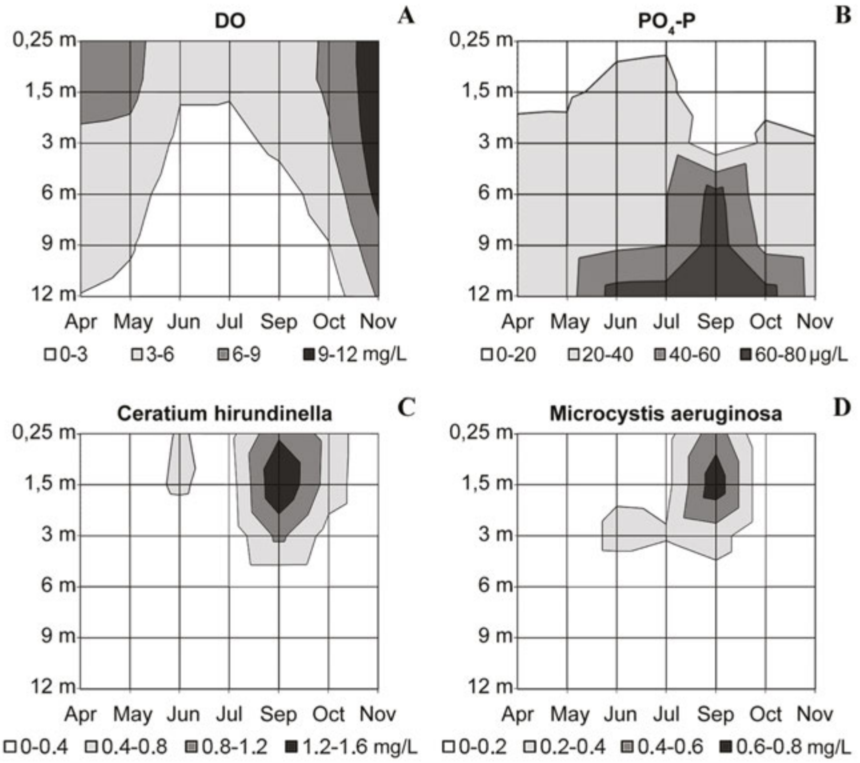

Figure 4. The dissolved oxygen (A), PO_4-P (B), biomass of *C. hirundinella* (C) and *M. aeruginosa* (D) at various depth in the lower region of the reservoir. *O teor de oxigénio dissolvido (A), PO_4-P (B), o* C. hirundinella *(C) e o* M. aeruginosa *(D) biomassa em diferentes profundidades na parte inferior (L) do reservatório.*

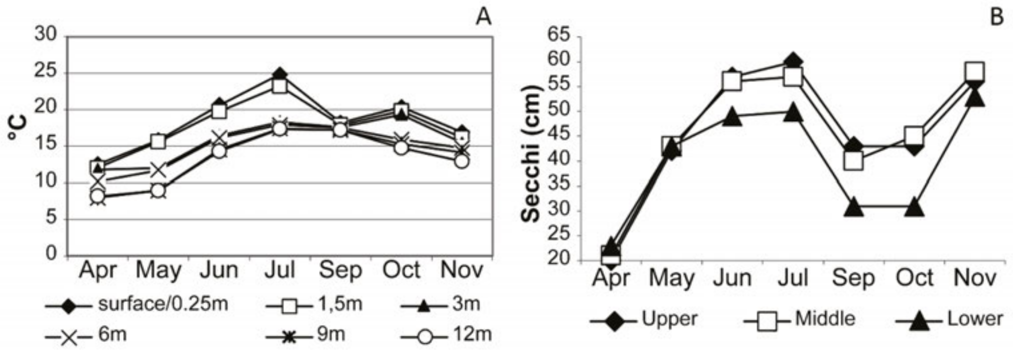

Figure 5. Temperature and transparency changes of the reservoir: A) temperature changes at various depths in the lower region of the reservoir (L); B) Secchi depth changes in the various regions of the reservoir. *Mudança da temperatura e transparência do reservatório: A) mudança de temperatura em diferentes profundidades na parte inferior do reservatório (L); B) mudanças de profundidade de Secchi em diferentes áreas do reservatório.*

and completely separated from the maximum temperature of the deeper layers. In September, the temperature of all layers decreased markedly to the same value at all layers (Fig. 5A). In October, the water temperature at the surface, at 1.5 m and at 3 m started to increase slightly, while it decreased in all the other layers. In November, the temperature of the entire water body decreased to nearly the same value.

The transparency of the water was the lowest in April at all three sampling sites (Upper, Middle, Lower), and then strongly increased in May (Fig. 5B). In June and July, the the transparency of the water at the sampling sites increased, while as a result of rainfall in September it reduced significantly (Fig. 5B). In October, the transparency of the water increased at the middle sampling site, however, while at the upper and lower sites it remained low. The transparency of the water in November increased strongly at all three investigated sites.

DISCUSSION

Based on our Canonical Variance Analysis (CVA) with the consideration of the chemical variables, it can be observed that the individual sampling sites and the associated two algae species are separated in the reservoir area. The reservoir was divided into three different parts. A discrete group is composed of those samples that originate from the upper part of the reservoir and during the year none of the two species occurred in this region (U). Samples from the middle part of the reservoir were grouped separately, i.e. locations where only *C. hirundinella* occurred (M), and the lower part of the reservoir (L) where the co-occurrence of *C. hirundinella* and *M. aeruginosa* was typical. The ammonium-ion concentration over the entire year was the highest at the sampling site where one none of the examined species was not occurred. Here we found the greatest change in the ammonium-ion concentration over the investigated period. The annual average of the ammonium-ion concentration was significantly lower at those sampling points where just the *C. hirundinella* has occurred. The lowest ammonium-ion concentration was found at those sampling sites where both investigated species was occurred. Considering the average for the whole year, the concentration of the orthophosphate-ion with the consideration of the whole yearly average was the highest at those sampling sites where none of the studied species were occurred. The annual average of orthophosphate ion concentration was significantly lower at those sampling sites where only *C. hirundinella* occurred. The annual average concentration of the orthophosphate ion was even lower – the lowest – at those sampling sites where both two species occurred. Here, the concentration of the orthophosphate ion varied most during the study period.

Based on our results in a mesotrophic reservoir, we identified an additional process: the lowest parts of the reservoir were characterised by the highest algal biomass, but lower nutrient concentrations, because the large quantities of living organisms used the nutrients and thus the amount of nutrients in the water body decreased.

The characteristic species composition and the community formed by the dominant species of a reservoir depend on meteorological, physical and chemical characteristics. During our study we were able to describe the potential mechanisms facilitating the common cyanobacterial and eukaryotic algal blooms (Fig. 6). We observed increases in algal biomass concentrations in September after a rainy, cold period. During this period, the reservoir experienced a brief period of non-stratification and parts of the hypolimnion became anoxic potentially resulting in the internal release of nutrients from the sediment. As these special hydrological and climatic circumstances allowed phosphorus to move up from the anoxic bottom region to the upper, photic layers, cyanobacterial and eukaryotic algal blooms occurred.

Considerable evidence suggests that nutrient conditions can facilitate the dominance of cyanobacteria. The explanations of the occurrence of Cyanobacteria taxa occurrence are quite obvious if the given Cyanobacteria taxa tend to have a competitive advantage in low N environments because such taxa are able to fix atmospheric N_2. Therefore, our data provides support for the hypothesis that low N concentrations favour cyanobacterial dominance if the available phosphorus is present. While nutrient concentrations were important in facilitating cyanobacterial development, the hydrologic regime of the lake may also have played an important role in the biomass increase of *C. hirundinella*. There may have been a positive feedback in the reservoir with increased biomass of cyanobacteria and more turbid conditions, which in turn, enhanced *C. hirundinella* growth.

CONCLUSION

Ecological communities are expected to be structured by a variety of stochastic and deterministic processes. Two deterministic processes are thought to play a major role in determining the coexistence of species in the same trophic level: interspecific competition and environmental filtering, where species are excluded from a community due to an inability to survive and reproduce in a given physical environment. The general rules for predicting the relative importance of

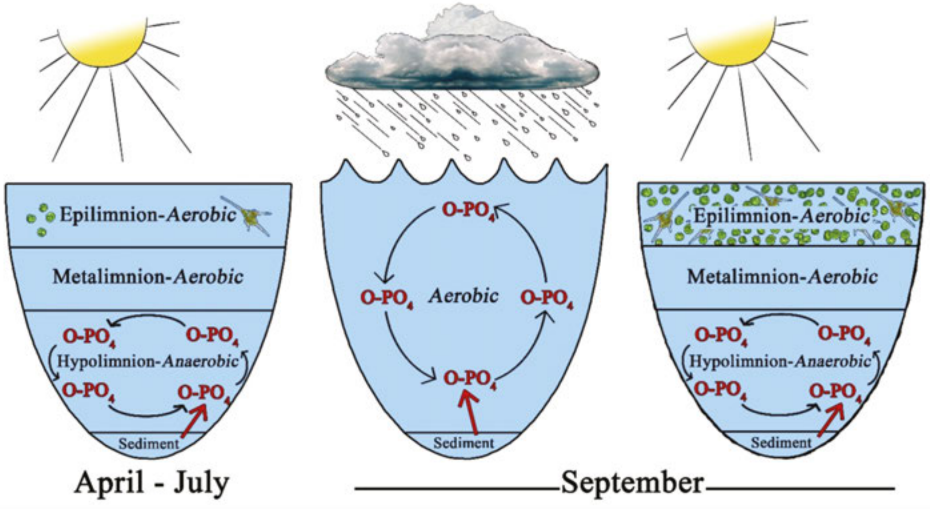

Figure 6. The cyanobacterial and eukaryotic algal bloom forming process in drinking water reservoir. *Formação da produção em massa de cianobactérias e algas eukarióticas no reservatório.*

these processes in different contexts are still largely unresolved. The results of this study strongly suggest that there is a positive association between the increase in the frequency of summer rainfall events, the resulting non-stratification and internal P-release, and cyanobacterial blooms. Therefore, identifying the causal factors related to cyanobacterial blooms is an important first step in managing the water quality problems of drinking water reservoirs. Low nitrogen levels in combination with internal phosphorus inputs from the watershed and sediment may have created conditions that were favourable for cyanobacterial production. Furthermore, mixing patterns helped to move up to the phosphorus up into the surface region to promote nitrogen-fixing cyanobacteria growth.

The complexity of the processes involved in the development of algal blooms cannot be completely understood with costly mesocosm or microcosm approaches. Therefore, it is extremely important to study and understand the phenomena that occur naturally in the reservoirs, and then use our experiences as a model on other water bodies. By doing so, we will be able to understand different ecosystem processes that control algal blooms and how they interact and respond to different inputs.

The co-existence of phytoplankton assemblages under similar environmental conditions has allowed the identification of functional groups made up of species with similar morphological and physiological characteristics. Fourteen groups of phytoplankton were identified in Reynolds's original study (Reynolds, 1980a,b). Some have been subdivided since, although the biggest change has been to re-label them (Reynolds, 1984). The Reynold's model includes such group – LM –, which together include the *C. hirundinella* and *M. aeruginosa*. The model will be redesigned, but these two species will remain in the given group. In some cases, the two species often occur together in large quantities in various types of standing water all over the world. At other times, from year to year one or the other become the dominant species in the same water. In addition, in many respects these two species have extremely different of ecological needs.

Cyanobacteria blooms are typically associated with eutrophic and poorly flushed waters (Paerl, 1988; Paerl *et al.*, 2001; Phieler *et al.*, 2009; Quin *et al.*, 2010; Smith. 1983). As surface waters become enriched with nutrients, particularly phosphorus (P), there is often a shift in the phytoplankton community towards dominance by cyanobacteria (Smith, 1983, Wang & Wang, 2009, Zhai *et al.*, 2009). The nutrient release from the sediment may have helped to further facilitate cyanobacterial blooms as has been reported in other waterbodies (Forbes *et al.*, 2008, Paerl 2009). Johnston & Jacoby (2003) also hypothesized that internal nutrient release was an important factor in fueling cyanobacterial blooms in a large lake in Seattle, Washington. Following the period of stratification, the lake began to mix as wind speed and rainfall events increased, and temperature gradients were minimal in the early fall (Wang & Wang, 2009).

Finally, this study demonstrated that higher temperatures coupled with elevated P concentrations frequently yielded growth rates of toxic *Microcystis* cells which exceeded all other treatments and populations.

Our study might also be used as a predictive tool supporting inter-disciplinary ecosystem management to assist in previewing the consequences of different management decisions. We suggest that management efforts should focus on controlling mesotrophic/eutrophic reservoirs. Because of the high amount of phosphorus released from the bottom of the water body due to anaerobic conditions, the phosphorus concentrated water needs to be released from the hypolimnion at intervals. This might be especially important during the summer months, before long-lasting rains to prevent undesirable cyanobacterial and/ or algal blooms or mass production.

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Biogeography and morphology of a poorly known diatom *Dorofeyukea rostellata* (Hustedt) Kulikovskiy & Kociolek

Zsuzsa Trábert[1,2,*], Krisztina Buczkó[1,3], Gábor Borics[4], Marija Gligora Udovič[5], Balasubramanian Karthick[6], Luc Ector[7], Angéla Földi[1,2], Keve T. Kiss[1] and Éva Ács[1]

[1] MTA Centre for Ecological Research, Danube Research Institute, 1113 Budapest, Karolina út 29, Hungary.
[2] Eötvös Loránd University, Doctoral School of Environmental Sciences, 1117 Budapest, Pázmány Péter sétány 1/A, Hungary.
[3] Hungarian Natural History Museum, Department of Botany, 1087 Budapest, Könyves Kálmán krt. 40, Hungary.
[4] MTA Centre for Ecological Research, Danube Research Institute, Department of Tisza River Research, 4026 Debrecen, Bem tér 18/c, Hungary.
[5] Department of Biology, Faculty of Science, University of Zagreb, 10000 Zagreb, Rooseveltov trg 6, Croatia.
[6] Biodiversity & Paleobiology Group, Agharkar Research Institute, G.G. Agarkar Road, Pune – 411004 India.
[7] Environmental Research and Innovation Department (ERIN), Institute of Science and Technology (LIST), 4422 Belvaux, 41 rue du Brill, Luxembourg.

[*] Corresponding author: trabert.zsuzsa@okologia.mta.hu

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ABSTRACT

Biogeography and morphology of a poorly known diatom *Dorofeyukea rostellata* (Hustedt) Kulikovskiy & Kociolek

Dorofeyukea rostellata is a rare and poorly known representative of *Dorofeyukea* genus. To date, this taxon has only been recorded only outside of Europe. In two Croatian lakes, we found a diatom in appearance similar to *Dorofeyukea rostellata* and within corresponding physical and chemical conditions. Due to the uncertain taxonomical position of this taxon, type material of *Dorofeyukea rostellata* and a closely related species, *Navicula grimmeioides*, were analysed by means of light and scanning electron microscopy. Upon a detailed investigation, we could clearly distinguish the Croatian population from *N. grimmeioides*. However, the Croatian population and the type material of *D. rostellata* differed only in minor characteristics. The most obvious difference is the shape of areolae. Nevertheless, differences between the shapes of the areolae can be caused by the degree of silicification, which often depends on the environmental conditions. Since almost every other characteristic of the Croatian population overlapped with that of the type material of *D. rostellata* we identified it as *D. rostellata*. Additionally, scanning electron microscopy analyses also revealed that *N. grimmeioides* belongs to the genus *Dorofeyukea*, so its new combination was proposed.

Key words: type material, *Dorofeyukea rostellata*, *Navicula grimmeioides*, calcareous habitat, new combination

RESUMEN

Biogeografía y morfología de una diatomea poco conocida Dorofeyukea rostellata *(Hustedt) Kulikovskiy & Kociolek*

Dorofeyukea rostellata *es un representante raro y poco conocido del género* Dorofeyukea*. Hasta la fecha, esta especie solo ha sido registrada fuera de Europa. En dos lagos croatas, encontramos una diatomea en apariencia similar a* Dorofeyukea rostellata *y en las condiciones físicas y químicas correspondientes. Debido a la incierta posición taxonómica de este taxón, el material tipo de* Dorofeyukea rostellata *y una especie estrechamente relacionada,* Navicula grimmeioides*, se analizaron mediante microscopía de luz y electrónica de barrido. Tras una investigación detallada, pudimos distinguir claramente la población croata de* N. grimmeioides*. Sin embargo, la población croata y el tipo de material de* D. rostellata *diferían solo en características menores. La diferencia más obvia es la forma de las areolas. Sin embargo, las diferencias pueden deberse al grado de silicificación, que a menudo depende de las condiciones ambientales. Como casi todas las demás características de* *la población croata se superponían con del material tipo de* D. rostellata*, lo identificamos como* D. rostellata*. Además, los análisis de microscopía electrónica de barrido también revelaron que* N. grimmeioides *también pertenece al género* Dorofeyukea*, por lo que se propuso una nueva combinación.*

Palabras clave: *ejemplares tipo,* Dorofeyukea rostellata*,* Navicula grimmeioides*, hábitat calcáreo, nueva combinación*

INTRODUCTION

Navicula grimmei var. *rostellata* Hustedt (1937, p. 236, fig. 13) was described from Indonesia in 1937 as a variety of *Navicula grimmei* Krasske. Kulikovskiy *et al.* (2019) transferred them into a newly erected genus *Dorofeyukea*, which was described on the basis of DNA sequence and morphological data as *Dorofeyukea rostellata* (Hustedt) Kulikovskiy & Kociolek and *Dorofeyukea grimmei* (Krasske in Hustedt) Kulikovskiy & Kociolek.

Distribution of *Dorofeyukea rostellata* is sporadic but wide and disparate (Fig. 1a); it was first recorded in the tropical equatorial lakes of Central Java and in warm springs in Central Sumatra (Hustedt, 1937). It was also recorded from Antarctica, in the sediment from King George Island (Kim & Park, 1988) as *Navicula grimmei* var. *rostellata*. The identity of the diatom documented by Kim & Park (1988) is ambiguous. However, the species found by Kim & Park is not *Dorofeyukea rostellata* based on the attached images according to Kim & Park's publication (1988). Consequently, we put it on the world map as formally noticed occurrence. Another occurrence of this taxon comes from Ghana (Smith *et al.*, 2015).

To study and assess the ecological potential of Croatian reservoirs, a country-wide survey was organised in 2016 and 2017. In some of the benthic samples, we found a diatom population similar to the members of genus *Dorofeyukea* with characteristic isodiametric areola (Potapova, 2013). We referred to it as 'Croatian population' in the followings. The most characteristic features of the taxon resembled *Dorofeyukea grimmei*. Consequently, we overviewed the taxa closely related to *Dorofeyukea grimmei*. Based on main characters we concluded that the reinvestigation of the original material is necessary to confirm the identification. The analyses of the type materials of *Dorofeyukea rostellata* and *Navicula grimmeioides* H.P. Gandhi in light (LM) and scanning electron microscopy (SEM) were carried out to clarify the identity of the Croatian diatom taxon. Our goal was to give an emended description of the ultrastructure of the studied taxa.

MATERIALS AND METHODS

Sampling

The Croatian standard diatom sampling methodology in water quality assessments of rivers and lakes was used in Croatia (Narodne Novine, 73/2013, 151/2014 and 78/2015). Sampling was performed in two artificial lakes Ričice and Tribalj following the "single habitat sampling" principle (Barbour *et al.*, 1999). As there were no stones as representative microhabitats at either of the sampling points, mud in the Lake Ričice (43.51065 °N, 17.11951 °E) and technolital (concrete) in Lake Tribalj (at 45.22876 °N, 14.66736 °E and 45.22651 °N, 14.67109 °E sampling points) were sampled on 22th July and 8th September in 2016, respectively (Fig. 1b). Five sub-samples were sampled at each sampling site. All samples were preserved with 4 % formaldehyde.

The following environmental variables were recorded at each sampling point during the sampling: water temperature (°C), pH, electric conductivity (µS/cm), dissolved oxygen (mg/L), oxygen saturation (%). Other variables were detected only in one point in each lake: alkalinity (mg/L), total hardness (mg/L), turbidity (NTU), BOD_5 (mg/L), COD-Mn (mg/L), NH_4–N (mg/L), NO_2–N (mg/L), NO_3–N (mg/L), Total N (mg/L), PO_{4-} P (mg/L), Total P (mg/L), Ca (mg/L), Mg (mg/L) and SiO_2 (mg/L) as per standard methods for water analysis (CEN, 2014). The measured values are presented in Table 1.

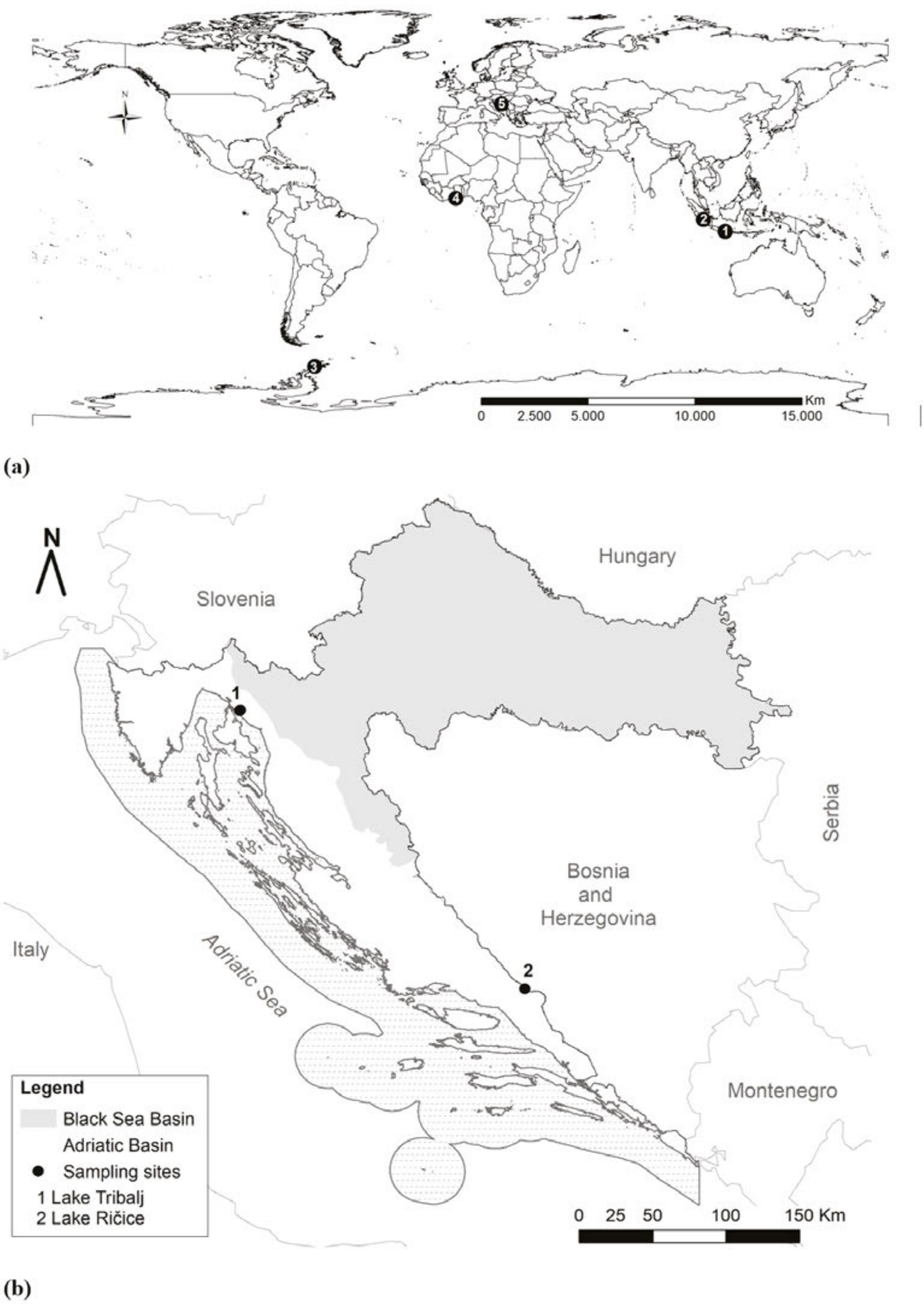

Figure 1. (a) World map distribution of *Dorofeyukea rostellata* (1 – Central Java, 2 – Central Sumatra, 3 – Antarctica (King George Island) (The species was recorded here but that must have been misidentification. However, we put it on the map because this occurrence of the species had formally been published.), 4 – Ghana, 5 – Lake Tribalj, Lake Ričice). *Distribución mundial de* Dorofeyukea rostellata*. (b) Sampling localities (1 - Tribalj and 2 - Ričice lakes, Croatia). Sitios de muestreo.*

Table 1. Physical and chemical variables in sampling sites on the sampling days. *Variables físicas y químicas en sitios de muestreo en los días de muestreo.*

	Ričice	Tribalj
Water Temperature (°C)	27.5	22.3
pH	7.66	7.33
Conductivity (µS/cm)	255	243
Dissolved oxygen (mg/L)	7.67	8.78
Oxygen saturation (%)	102.3	102.3
Alkalinity (mg/L)	140.1	118
Total hardness (mg/L)	150.2	149
Turbidity (NTU)	8.35	11.7
BOD_5 (mg/L)	0.93	1.5
COD–Mn (mg/L)	1.25	2
NH_4–N (mg/L)	0.075	0.041
NO_2–N (mg/L)	0.024	0.003
NO_3–N (mg/L)	0.155	0.19
Total N (mg/L)	0.892	0.41
PO_4–P (mg/L)	0.0014	0.0031
Total P (mg/L)	0.003	0.028
Ca (mg/L)	50.26	48.3
Mg (mg/L)	5.88	6.77
SiO_2 (mg/L)	3.35	3.6

Microscopic investigations

In the laboratory, samples were rinsed with distilled water to remove formaldehyde and subsequently treated with hot H_2O_2 and mounted in Naphrax®. Slides were analysed with the Olympus IX-70 light microscope (1500 ×). At least 500 frustules were counted for the Croatian samples.

Using LM 46 valves of *Dorofeyukea rostellata* were documented in the type material and further 9 specimens in SEM. The type material of *Navicula grimmeioides* was observed with an Olympus BX53 DIC microscope equipped with Olympus DP73 camera and cellSens 1.13 imaging software.

A part of the cleaned and washed samples was filtered through a 3 µm Isopore™ polycarbonate membrane filter (Merck Millipore, Germany) for SEM studies. The filter was fixed onto a stub using double-sided carbon tape and coated with gold using a rotary-pumped spatter coater Quorum Q150R S (ZEISS). Fine structures of diatom frustules were observed with Zeiss EVO MA 10 SEM (ZEISS) operated at 10 kV and 10 mm distance using SEM detectors in case of Croatian samples and type material of *Dorofeyukea rostellata*. For scanning electron microscopy of type material of *Navicula grimmeioides*, processed samples were air dried on the cover glass and pasted over the aluminium stubs using double-sided carbon tape. Samples were gold coated for approximately 3 minutes using an Emitech K575X sputter coater and observed with a Zeiss EVO 50 Lab 6 Scanning Electron Microscope. Observations were made under 15–20 kV acceleration voltages at a 5–8 mm Working Distance. Micrographs were recorded using Zeiss SmartSEM software. Gandhi's samples used in this study are housed at Agharkar Research Institute's Diatom Herbarium, Pune. All statistical analyses were performed in R version 3.4.2 implemented in Windows. Maps were generated using ArcMap 10.1.

Type material of *Dorofeyukea rostellata* investigated: AS 745 (lectotype), 7.3.1929 (under the name *Navicula grimmei* var. *rostellata*).

LM images were taken from the powder of the lectotype material washed before mounting into Naphrax®.

The studied slides of the Croatian population of *Dorofeyukea rostellata* are deposited in the Hungarian Natural History Museum BP-NHMUS-ALG 2300-2305.

RESULTS

Investigation of the Croatian population (Figs. 2-13, Figs. 38-57)

In LM the outline of symmetrical valves is lanceolate with slightly capitate or broadly rounded apices (Figs. 2-13). The valve length is 12.8-30.8 µm (mean = 18±4.0 µm, n = 26) and the width is 5.3-7.3 µm (mean = 6.1±0.5 µm, n = 26). Striae are radial, (slightly) convergent on the apices. The number of striae is 19-25 in 10 µm (mean = 22±1.3, n = 26). Central area is slightly asymmetric bowtie-shaped. Axial area is narrow. Raphe is filiform. There is no stigma on the surface of the valve.

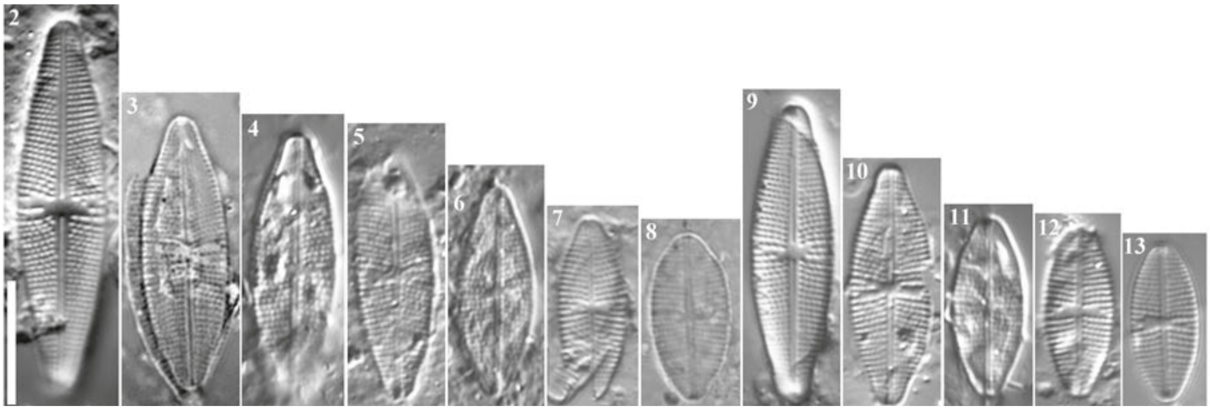

Figure 2-13. *Dorofeyukea rostellata* found in the Croatian lakes. LM: (Figs 2-8) from Ričice, (Figs 9-13) from Tribalj. Scale bar = 10 µm. *Especies encontradas en los embalses de Croacia. MO: (Figs 2-8) de Ričice, (Figs 9-13) de Tribalj. Barra de escala = 10 µm.*

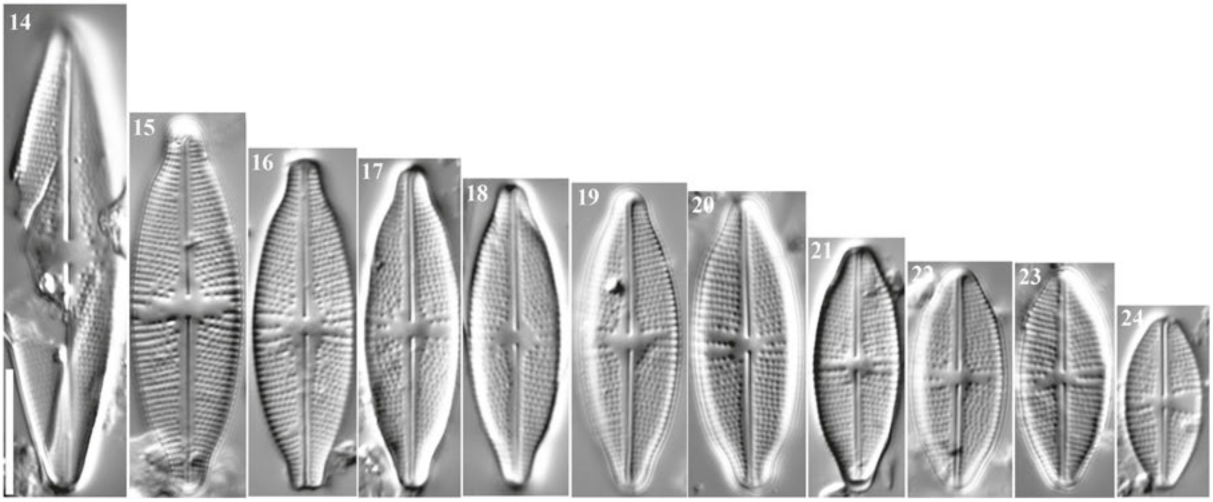

Figure 14-24. *Dorofeyukea rostellata*. LM micrographs of the type material. Scale bar = 10 µm. Dorofeyukea rostellata*. Micrografías MO del material tipo. Barra de escala = 10 µm.*

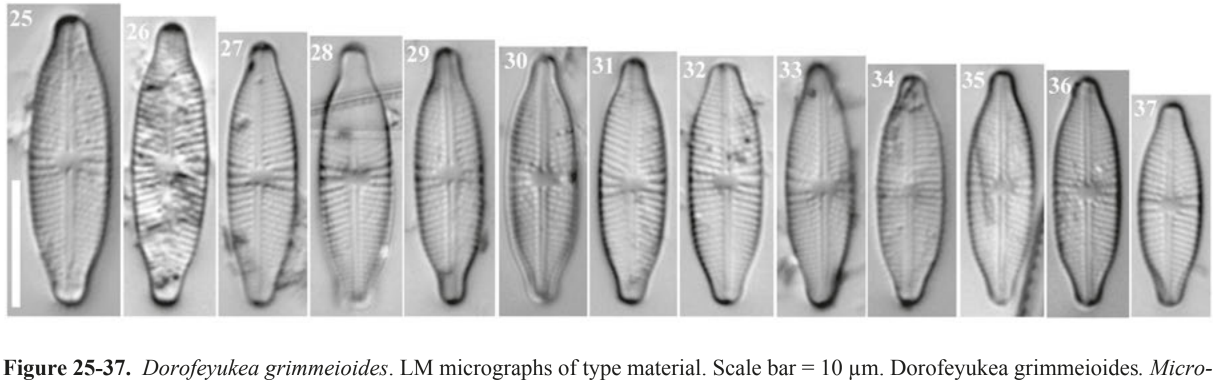

Figure 25-37. *Dorofeyukea grimmeioides*. LM micrographs of type material. Scale bar = 10 µm. Dorofeyukea grimmeioides*. Micrografías MO del material tipo. Barra de escala = 10 µm.*

In SEM (Figs. 38-57), striae are composed of single rows of elongated areolae, which continue on the mantle in several rows (Figs. 38, 41, 43). On the central area there are (1)2-3(4) striae creating an irregular hyaline field (Figs. 40, 42, 44, 56). In the middle part of the valve the interstriae can be wider or narrower (Figs. 41, 42, 44, 45). Externally, the areolae are rectangular, occasionally transversally elongated at the central part (Fig. 55) and slit-like on the apices (Figs. 52-54). The proximal raphe fissures are straight and slightly drop-shaped.

Internally, the areolae are closed by hymens (Fig. 57). Most of them are also elongated, some of them are dot shaped (Figs. 56, 57). Number of areolae is (24) 25-30 in 10 µm (mean = 27.7±1.9,

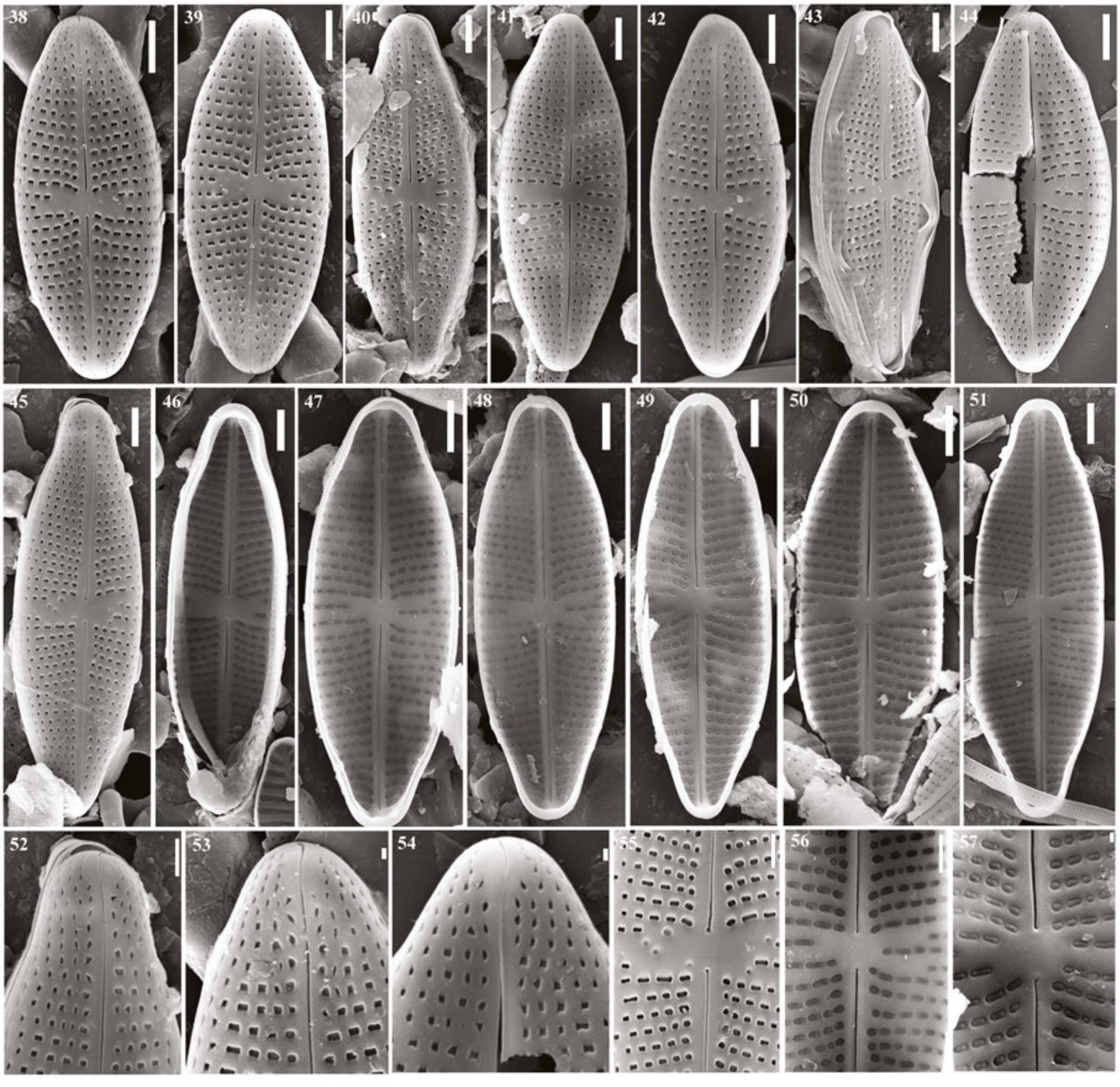

Figure 38-57. *Dorofeyukea rostellata* found in the Croatian lakes. SEM micrographs from Tribalj Lake. Scale bar = 2 µm for Figs 38-51; 1 µm for Figs 52, 55, 56 and 200 nm for Figs 53, 54, 57. *Especies encontradas en los lagos croatas. Fotografías MEB del lago Tribalj. Escala = 2 µm para las figuras 38-51; 1 µm para las figuras 52, 55, 56 y 200 nm para las figuras 53, 54 y 57.*

n = 14). The axial area is relatively broad (Figs. 38-51). Internally, the raphe fissures are straight, terminating in small helictoglossae near the poles (Figs. 46-51). Narrow pseudoseptum is present on both apices (Figs. 46-51).

Investigation of type material of *Dorofeyukea rostellata* (Hustedt) Kulikovskiy & Kociolek (Figs. 14-24, 58-67)

In LM the valve is lanceolate with capitate or rostrate apices. The length of the valve is 14.6-39.1 µm (mean = 24.1± 4.5 µm, n = 55), the width is 6.6-10.4 µm (mean = 8.1± 0.8 µm, n = 55). Striae are radial, becoming slightly convergent on the apices. Number of striae is 17-25 in 10 µm (mean = 20.2±1.4, n = 55) and it can be denser toward the ends. Areolae are thin and drop-like and can easily be counted in LM. The number of areolae is 20-32 in 10 µm (mean = 24.5±2.4, n = 55). The raphe is straight. There is no stigma on the valve surface.

In SEM externally, the areolae are transapically slit-like continuing on the mantle (Fig. 66). Internally, the areolae are mostly round, some of them are oval; they are closed by hymen (Figs. 63-65, 67). The striae can be shorter or longer near the central area and become denser toward the ends. Striae are radial, slightly convergent on the apices. The axial area is relatively broad and become wider near the field of central area (Fig. 63). Raphe is straight; externally, distal raphe endings are bent to the same side (Figs. 58-62). The proximal raphe fissures are straight and slightly drop-shaped on the outer valve (Fig. 66), dot-like on the inner valve (Fig. 67). Internally, the distal raphe endings are straight, terminating in small helictoglossae near the poles (Figs. 63-65). Central area is transapically oval outside and fairly irregular inside.

Holotype: Ground slime of Telaga Pasir, middle Java. Isotype: warm springs of Kadjaj, central Sumatra. Hustedt F. 1937. *Arch. Hydrobiol. Suppl.* 15: 236, pl. 17, fig. 13. QH90A67s, Figured also in Schmidt's Atlas, pl. 405, figs 30-34.

Lectotype: N3/45. Sumatra. Warme Quelle v. Kadjaj. SKW2bα, Simonsen, R. 1987. Atlas and catalogue of the diatom types of Friedrich Hustedt: 201, pl. 310, figs 6-10 (this material was investigated and illustrated in our study).

Isolectotype: N3/46. Sumatra. Warme Quelle v. Kadjaj. SKW2bα, Simonsen, R. 1987. Atlas and catalogue of the diatom types of Friedrich Hustedt: 201.

Investigation of type material of *Navicula grimmeioides* H.P. Gandhi (Figs. 25-37, 68-81)

In LM, the valve outline is lanceolate with capitate or rostrate apices. The length of the valve is 16.8-22.6 µm (mean = 18.7±2.0, n = 20), the width is 5.2-6.1 µm (mean = 5.4±0.4, n = 20). Striae are radial throughout the valve, their number is 18-26 in 10 µm (mean = 21.4±2.7, n = 20). They are composed of observable but hardly to analyse coarse areolae. Central area is round. The raphe is straight. There is no stigma on the valve.

In SEM, externally, the areolae are transapically elongated, thin or slit-like (Figs. 77, 78). The number of areolae is 25-35 in 10 µm (mean = 32.1±3.2, n = 10). They are arranged in single rows of striae that continue on the mantle (Figs. 78, 81). Central area is round. Axial area is relatively broad and irregular. Raphe runs straight with bending distal endings. The proximal raphe fissures are strongly drop-shaped (Fig. 72).

Internally, the areolae are mostly round, some are oval which are closed by a hymen (Figs. 69, 70, 73, 79-81). The distal raphe endings are straight (Figs. 77, 78), terminating in small helictoglossae near the poles (Fig. 79). Proximal raphe fissures are slightly deflected to the same side (Figs. 74, 75).

Lectotype (designated here):—[illustration] Figs. 73–75 in p. 41, pl. 2 in Gandhi (1998: 324).

Type material: Lake Chandola border side with *Ceratophyllum*, Ahmedabad, India. Sample collected by H.P. Gandhi on 17 April 1960. Gandhi labelled this sample as "Sr-185 Ahmedabad: Lake Chandola border side with *Ceratophyllum*, a further spot cleaned 17-4-1960". Gandhi, H.P. (1998). Fresh-water Diatoms of Central Gujarat. Dehra Dun, India: Bishen Singh Mahendra Pal Singh, pl. 2, figs. 73-75.

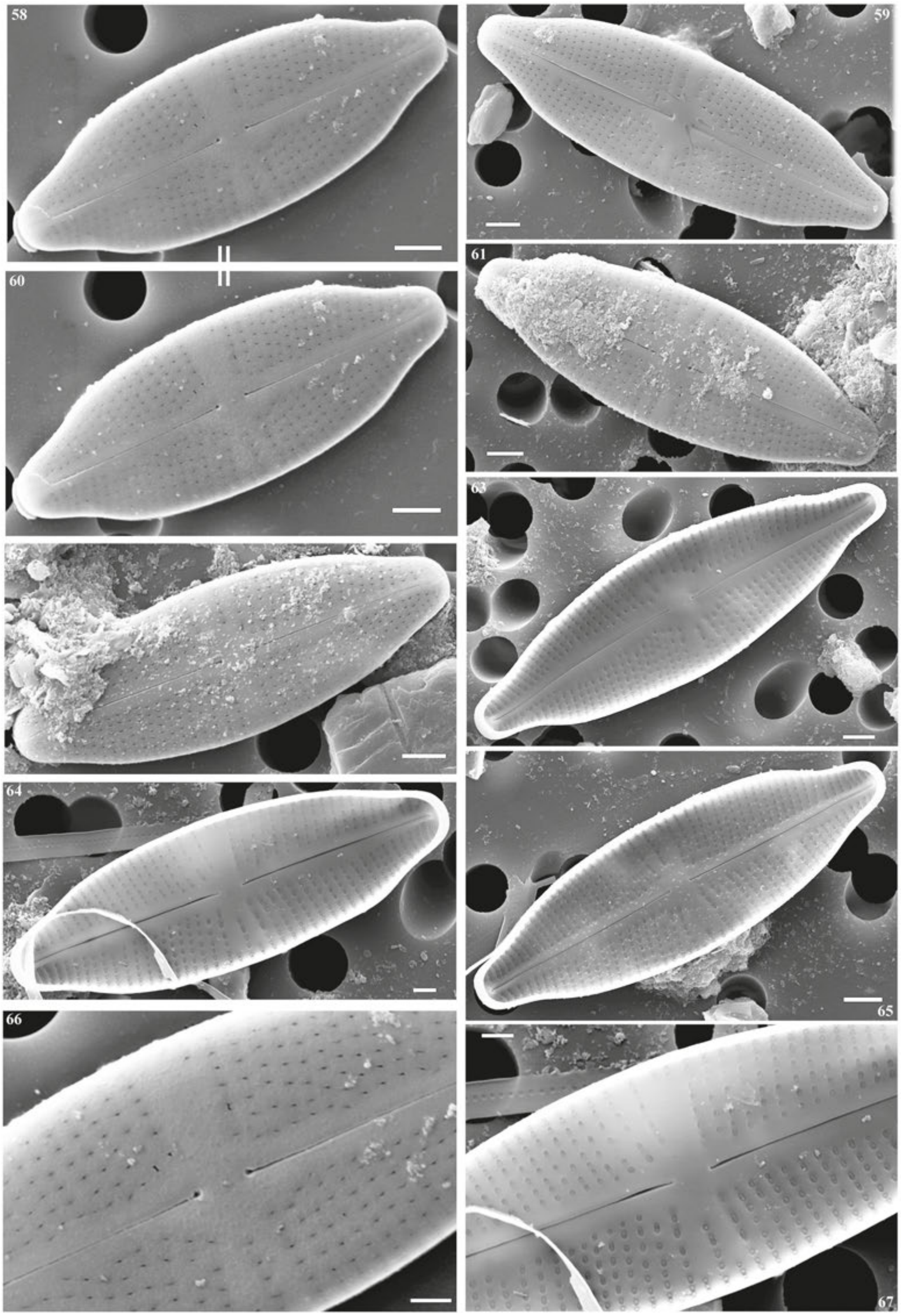

Figure 58-67. *Dorofeyukea rostellata*. SEM micrographs of the type material. Scale bar = 2 µm for Figs 58-63, 65; 1 µm for Figs 64, 66, 67. Dorofeyukea rostellata. *Micrografías MEB del material tipo. Barra de escala = 2 µm para las figuras 58-63, 65; 1 µm para las figuras 64, 66, 67.*

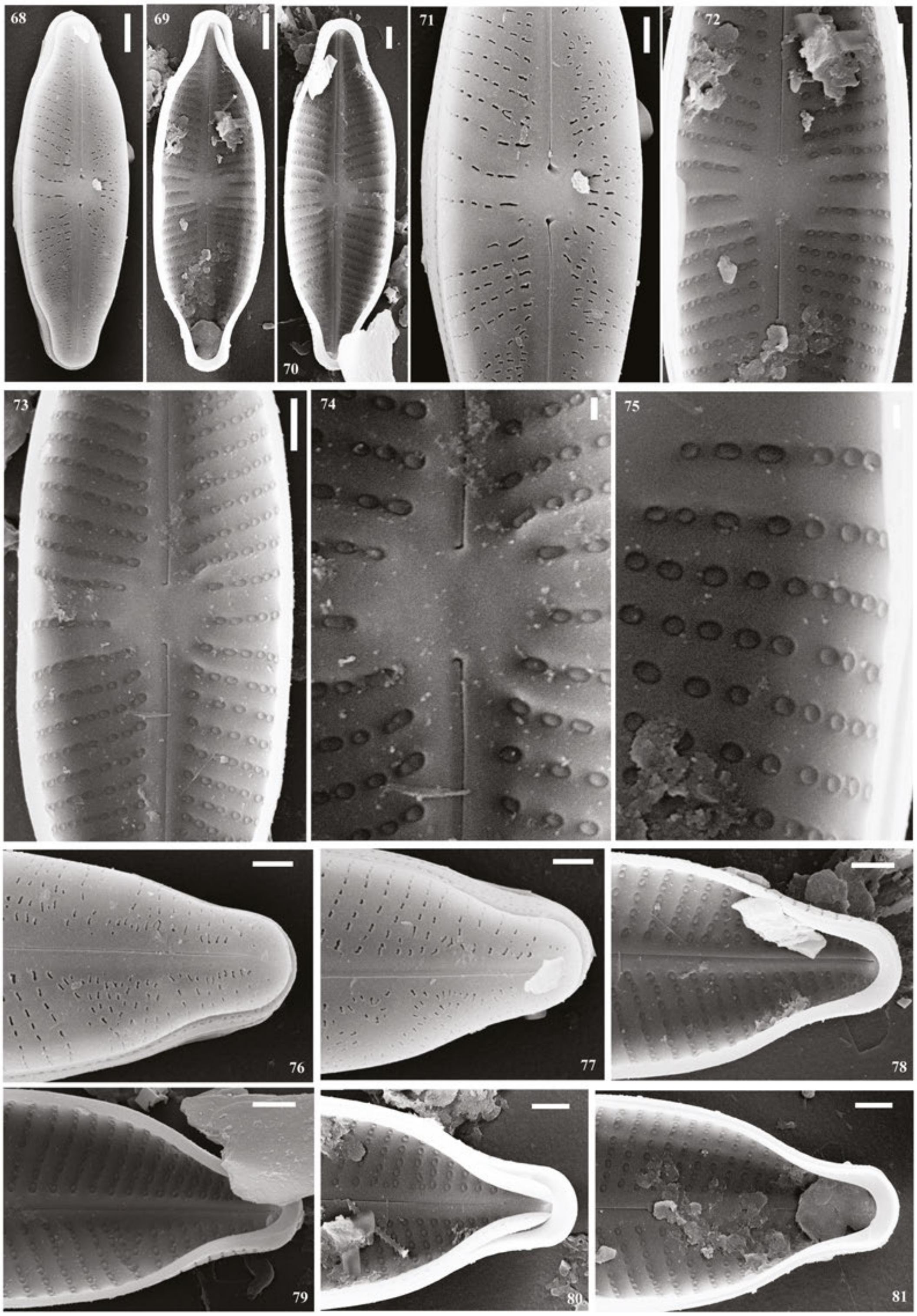

Figure 68-81. *Dorofeyukea grimmeioides*. SEM micrographs of the type material. Scale bar = 2 µm for Figs 68, 69; 1 µm for Figs 71-73, 76-81; 200 nm for Figs 74, 75. Dorofeyukea grimmeioides*. Micrografías MEB del material tipo. Barra de escala = 2 µm para las Figuras 68, 69; 1 µm para las figuras 71-73, 76-81; 200 nm para las figuras 74, 75.*

Habitat and ecology

Dorofeyukea rostellata populations were found in two Croatian lakes with similar physical and chemical characteristics (Table 1). Both lakes were oligotrophic, oligosaprobic and situated in calcareous bedrock; COD values were below 2 mg/L and oxygen saturation was always more than 85 %. The water of the lakes is neutral to slightly alkaline with pH 7.66 (Ričice), 7.12 and 7.53 (Tribalj1, Tribalj3, respectively) and with low conductivity (255 µS/cm for Lake Ričice; 241 µS/cm for Lake Tribalj1 and 243 µS/cm for Lake Tribalj3). The studied taxon occurred in the northern and in the eastern part of Tribalj, and in the northern part of Ričice. They occurred with low relative abundances (0.96 % in Lake Ričice, 1.68 % in the eastern part and 0.49 % in the northern part of Lake Tribalj). *Achnanthidium straubianum* (Lange-Bertalot) Lange-Bertalot was the only species that reached relatively high abundance in all three samples, it was subdominant in Lake Ričice (4.34 %) and dominant in Lake Tribalj with 6.49 % and 10.73 % of relative abundance in northern and in the eastern part of the lake. In Lake Ričice the dominant species were *Amphora inariensis* Krammer (36.14 %), *Cavinula cocconeiformis* (W. Gregory ex Greville) D.G. Mann & Stickle in Round *et al.* (10.12 %) and *Pantocsekiella ocellata* (Pantocsek) K.T. Kiss & Ács (26.02 %). In Lake Tribalj the dominant species were *Achnanthidium minutissimum* (Kützing) Czarnecki (north part 18.03 % and east part 21.46 %), *Encyonopsis subminuta* Krammer & E. Reichardt (north part 12.74 % and east part 15.85 %) and *Ulnaria delicatissima* (W. Smith) Aboal & P.C. Silva (north part 11.3 % and east part 16.59 %).

DISCUSSION

Comparing the Croatian population with the type material of *Dorofeyukea rostellata* revealed differences in valve, central area and the areola shape, and the width and shape of the axial area. In SEM it can be seen that areolae of the Croatian population are mostly rectangular or square shaped in the middle (Fig. 55) and perpendicularly elongated toward the ends (Figs. 52-54) in external view, while that of *D. rostellata* are transapically slit-like (Figs. 58-62, 66). Furthermore, the axial area of the Croatian population is narrow and regular (Figs. 38-51), whereas it is wide and irregular in case of the type material of *D. rostellata* (Figs. 60, 63-65). However, as they overlapped with each other in the most important characteristics: valve apices and outline, striation, stria and areola density (Fig. 82), we cannot distinguish them as distinct taxa.

The presented Croatian population of *Dorofeyukea rostellata* resembles *Navicula grimmei* Krasske, a species described from Hessen, Germany (Krasske, 1925: 45, fig. 1: 14). *Navicula grimmei* had been synonymized to *Navicula kotschyi* (Krasske) Grunow (1860: 538, fig. 2: 12) by Krammer & Lange-Bertalot (1986). *Navicula kotschyi* was also transferred to *Dorofeyukea* genus by Kulikovskiy *et al.* (2019). *Dorofeyukea kotschyi* (Grunow) Kulikovskiy, Kociolek, Tusset & T.A.V. Ludwig differs from the Croatian population because *D. kotschyi* has more capitate ends and the areola density is 20-24 in 10 µm (Tyree & Bishop, 2015), while the Croatian population has denser areolae, 24-30 in 10 µm (Table 2). Furthermore, the areolae of *D. kotschyi* are uniform throughout the valve (Tyree & Bishop, 2015), whereas they are narrow and transapically elongated toward the apices in the Croatian population. Kulikovskiy *et al.* (2019) ignored that *Navicula grimmei* was synonymized to *Navicula kotschyi* and they transferred the species into *Dorofeyukea* genus. But they used an incorrect reference as its basionym, so *Dorofeyukea grimmei* (Krasske in Hustedt) Kulikovskiy & Kociolek is invalid at this moment according to the ''International Code of Nomenclature for Algae, Fungi and Plants" (McNeill *et al.*, 2012). Kulikovskiy *et al.* (2019) described a new species *Dorofeyukea indokotschyi* Kulikovskiy, Maltsev, Andreeva & Kociolek, which is a closely relative of *D. kotschyi*; but *Dorofeyukea indokotschyi* has definitely more capitate ends than the Croatian population.

The Croatian population differs from *Navicula grimmeioides* (Gandhi, 1998: 324, fig. 41: 73-75) by the following characteristics: valve apices, shape of the central area, striation and areola shape. The Croatian population has less

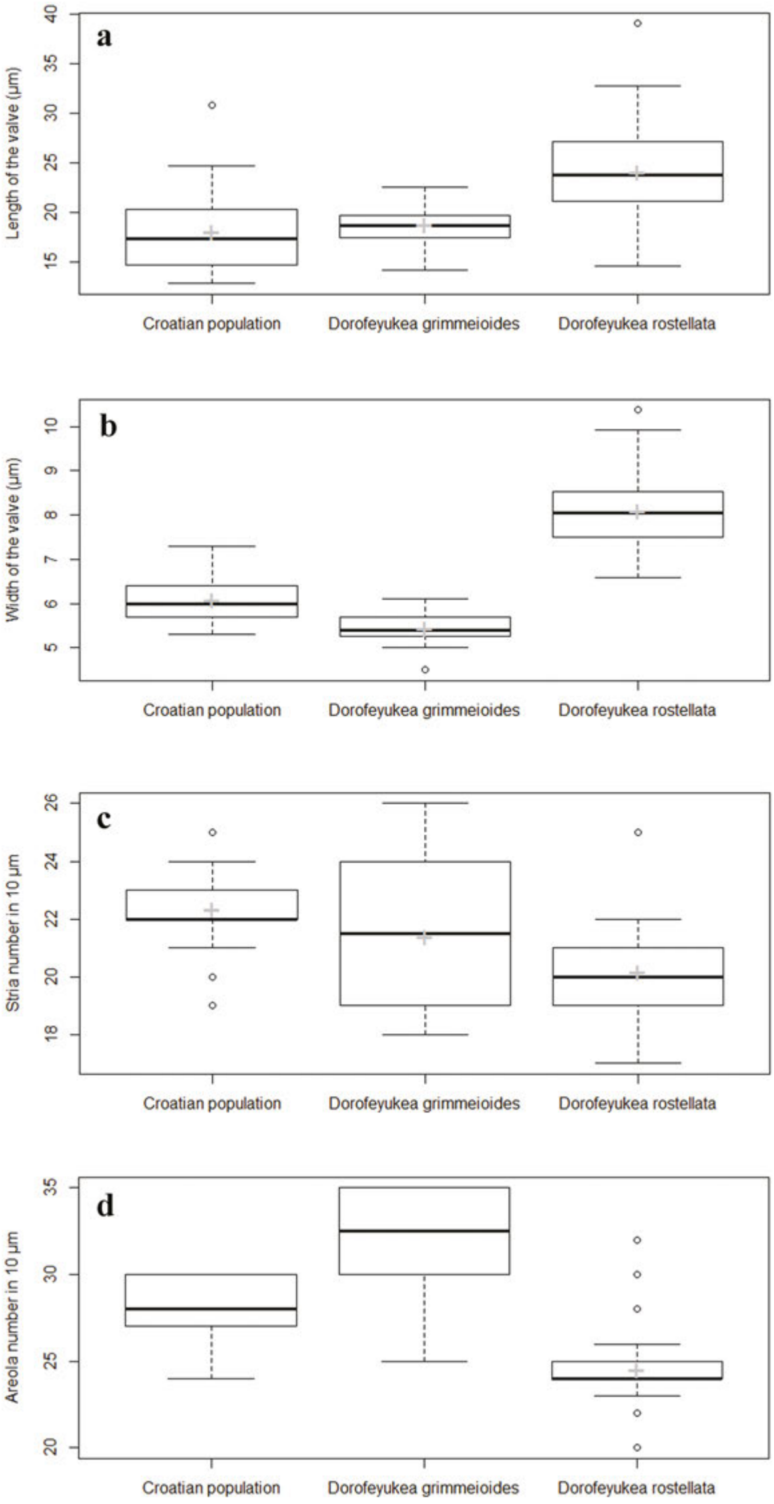

Figure 82. Box plots of length (a), width (b), striae (c) and areola number (d) in 10 µm of the two investigated type material and the Croatian population. The grey cross on the box marks the mean of the metrics. *Diagramas de cajas de longitud (a), ancho (b), estrías (c) y areola (d) en 10 µm de los dos materiales investigados y la población croata. La cruz gris en el cuadro marca la media de las métricas.*

Table 2. Differential diagnosis of the Croatian population and related taxa. *Diagnóstico diferencial de la población croata y taxones relacionados.*

	Length (µm)	**Width (µm)**	**Striae in 10 µm**	**Areolae (shape and number in 10 µm)**	**Valve outline**	**Axial area**	**Raphe**	**Central area**	**Biogeography**	**References**
Croatian population	12.8-30.8	5.3-7.3	19-25 radial slightly convergent on the apices	(24)25-30 elongated in single rows which continue on the mantle; externally rectangular, transapically elongated at the central part and slit-like on the apices	lanceolate with slightly capitate or broadly apices	narrow	filiform; raphe fissures are straight, internally terminating in small helictoglossae near the poles	slightly asymmetric bowtie shape	Tribalj and Ričice, Croatia	present study
Dorofeyukea ancisa (Hustedt) Kulikovskiy & Kociolek (Hustedt 1953: 48, pl. 150, fig. 9)	14	5-6	20 radiate, at apices nearly parallel	NA	rectangular with little convex edges	narrow	filiform	small, transapically slightly widened	Oasis Gafsa, southern Tunisia	Hustedt (1953)
Dorofeyukea grimmei (Krasske in Hustedt) Kulikovskiy & Kociolek (Krasske 1925, p. 45, pl. 1, fig. 14)	18-21	6	~25 the two middle shortened and slightly coarser; towards the ends become narrower	quite coarse (20-24)	elliptic-lanceolate with capitate apices	narrow	NA	square	Laurentian Great Lakes, North America	Hustedt (1930), Potapova (2013)
Dorofeyukea grimmeioides (H.P. Gandhi) Ács, Buczkó & Ector comb. nov. (Gandhi 1998: 324, p. 41, figs 73-75)	14.2-22.6	4.5-6.1	18-26 radial	25-35 thin, transapically elongated, slit-like; closed by hymen in the inner valve	lanceolate with capitate or rostrate apices	relatively broad	straight, proximal raphe fissures strongly drop-shaped	round, forming narrow stauroid fascia with 3 shortened striae	Gujarat, India	Gandhi (1998), present study
Dorofeyukea indokotschyi Kulikovskiy, Maltsev, Andreeva & Kociolek (Kulikovskiy *et al.* 2019: figs 2-4)	25-26	7-7.5	20-21 weakly radiate, almost parallel at the poles	rectangular	elliptic to linear elliptic, ends protracted, abruptly short-rostrate	narrow	filiform, central pore small, appearing as puncta	narrow stauroid fascia surrounded by 1-3 irregularly shortened striae	Indonesia, Papua, Lake Sentani	Kulikovskiy *et al.* (2019)
Dorofeyukea ivatoensis (Metzeltin & Lange-Bertalot) Kulikovskiy & Kociolek (Metzeltin & Lange-Bertalot 2002: 286, pl. 27, figs 6-10, pl. 28, figs 1-3)	18-34	6.6-9.5	21-24 radiate, proximally becoming subparallel, finally slightly convergent near the poles; stigma in the central area absent	15(20) rather coarse, some moderately elongated transapically in the middle, becoming smaller and denser towards the ends; isodiametric (punctiform) to apically elongated (only in SEM observed), the areolae form continuous rows from the valve face over the mantle	elliptic, ends shortly protracted broadly, more or less subcapitate, finally broadly rounded	linear, narrow to very narrow, not or very little broadened to the middle	filiform, straight with inconspicuous central pores, broadly deflected	slightly variable to barely developed; mostly small, circular or elliptic	"Island Continent", Madagascar	Metzeltin & Lange-Bertalot (2002)

Cont.

Table 2. (cont.)

Dorofeyukea kotschyi (Grunow) Kulikovskiy, Kociolek, Tusset & T.A.V. Ludwig (Grunow 1860: 538, pl. 2: fig. 12)	18-19 18.0-25.3	5-5.5 6.0-8.1	20-24 radiate, at apices nearly parallel radiate	coarse and visible in LM externally round, internally transapically linear	linear with parallel central valve margins and distinctly capitate apices	narrow and linear	filiform with unilaterally deflected distal ends	bowtie-shaped with marginal areolae	Laurentian Great Lakes, North America	Grunow (1860), Potapova (2013), Kulikovskiy *et al.* (2019)
Dorofeyukea orangiana (R.M. Patrick) Kulikovskiy & Kociolek (Patrick 1959: 97, pl. 8, fig. 4)	17-22	5-7	18-20 (towards the ends 26) radiate, distinctly punctate	20-25 (Potapova 2013)	linear-lanceolate with narrow, rostrate, rounded ends	narrow, widening toward the central area	proximal raphe fissures strongly drop-shaped	rounded (more or less)	Sabine River, Texas, Lake Maurepas, Lousiana, Ridley Creek, Pennsylvania, Patuxent River, Maryland	Patrick (1959), Potapova (2013)
Dorofeyukea rostellata (Hustedt) Kulikovskiy & Kociolek (Hustedt 1937: 236, pl. 17, fig. 13)	14.6-39.1	6.6-10.4	17-25 radial, slightly convergent on the apices	20-32 externally slit-like	lanceolate with capitate or rostrate apices	broad	straight, proximal raphe fissures slightly drop-shaped	transapically oval on the outer and irregular on the inner valve, forming narrow stauroid fascia with 2-3 irregularly shortened striae	Java, Bali, Sumatra	Hustedt (1937), and own measurements
Dorofeyukea savannahiana (R.M. Patrick) Kulikovskiy & Kociolek (Patrick 1959: 97, pl. 8, fig. 7)	17-22	4-5	20-22	24-28 (Potapova 2013) radiate, distinctly punctate	linear with distinctly capitate ends	narrow	straight, proximal raphe fissures slightly drop-shaped	transverse, not reaching the margin of the valve	Savannah River, United States	Patrick (1959), Potapova (2013)
Dorofeyukea texana (R.M. Patrick) Kulikovskiy & Kociolek (Patrick 1959: 98, pl. 8, fig. 5)	18-22	5-7	18-21	20-27 (Potapova 2103) irregularly shortened on each side of the central nodule; parallel at apices of the valve, otherwise radiate; distinctly punctate	margins are parallel with narrowed, distinctly rostrate ends	narrow	straight with slightly unilaterally deflected proximal ends	transverse	Guadalupe River, United States	Patrick (1959), Potapova (2013)

capitate apices, especially the smaller specimens (Figs. 55, 56), than *N. grimmeioides*. Striae of the Croatian population are radial but become convergent on the apices (Figs. 2-13). Furthermore, its striae can be longer or shorter in the central part (Figs. 40-42) forming a bowtie shape central area, while in the case of *N. grimmeioides* striae are radial throughout the valve and the central area is round (Figs. 25-37, 73-75). In SEM the Croatian population has rectangular areolae (Figs. 55-57), which become lineolate-like toward the apices (Figs. 52-54) whilst that of in case of *N. grimmeioides* has transapically slit-like areolation (Figs. 68-70). Externally, the proximal raphe ends of the Croatian population are dot-like fissures (Figs. 38-45, 55) while drop-shaped in case of *N. grimmeioides* (Fig. 72).

Based on the description of *Dorofeyukea* genus (Kulikovskiy *et al.*, 2019), *N. grimmeioides* also belongs to *Dorofeyukea*, because its central area forming narrow stauroid fascia (Figs. 68-70), surrounded with 2-3 shortened striae, its areolae covered internally by dome-shaped hymens (Figs. 74, 75), distal raphe ends hook-shaped going to mantle (Fig. 76), and it has pseudoseptum at distal ends (Figs. 69, 70, 78-81). Judging by morphological characteristics, we propose the transfer of *N. grimmeioides* to the genus *Dorofeyukea* as follows:

Dorofeyukea grimmeioides **(H.P. Gandhi) Ács, Buczkó & Ector comb. nov.**

Basionym: *Navicula grimmeioides* H.P. Gandhi 1998, Bishen Singh Mahendra Pal Singh. Dehra Dun. India 324, p. 41, pl. 2, figs. 73-75.

Some other species, as *Dorofeyukea texana* (R.M. Patrick) Kulikovskiy & Kociolek (Patrick 1959: 98, fig. 8: 5) also resembles the Croatian population, but its areolae on the central part are not elongated transversally. *Dorofeyukea savannahiana* (R.M. Patrick) Kulikovskiy & Kociolek (Patrick 1959: 97, fig. 8: 7) have more capitate ends. According to Potapova (2013), it is possible that *Dorofeyukea kotschyi* is rather variable in valve outline, shape and density of areolae, and width of the pseudoseptum and both *D. texana* and *D. savannahiana* are conspecific with it. Kulikovskiy *et al.* (2019) refer them as valid species. Potapova (2013) was not able to distinguish pseudoseptae in *D. savannahiana*. Narrow pseudoseptum is present on both apices in case of the Croatian population. *Dorofeyukea orangiana* (R.M. Patrick) Kulikovskiy & Kociolek (Patrick 1959: 97, fig. 8: 4) and *Dorofeyukea ivatoensis* (Metzeltin & Lange-Bertalot) Kulikovskiy & Kociolek (Metzeltin & Lange-Bertalot 2002: 286, figs. 27: 6-10, 28: 1-3) have round central area. In addition, the central area of *D. ivatoensis* is small and its distal raphe end is curved. The central area of *Dorofeyukea ancisa* (Hustedt) Kulikovskiy & Kociolek (Hustedt 1953: 150, fig. 9) is smaller than that of Croatian population (Table 2).

Our study pointed out that investigation of the type materials can be very important, particularly in case of rare taxa. We also want to stress here that the intensive studies on diatom taxonomy using high-resolution microscopic techniques, for better understanding of the ultrastructure of diatom frustule, can lead to new species description or combination of species. Although SEM analyses also revealed that the studied taxa do not belong to the genus *Navicula* sensu stricto, molecular data are important for erecting a new genus.

SUMMARY

Dorofeyukea rostellata is a rare diatom, known from various habitats. Here we reported the first European occurrence of this diatom. Detailed ultrastructural investigations of the type material of *Navicula grimmeioides*, which resemble to *Dorofeyukea rostellata*, revealed that its characteristics fit the main features of the genus *Dorofeyukea*, so we proposed to transfer it into the genus *Dorofeyukea* as a new combination.

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Ceratium furcoides (Levander) Langhans in reservoirs at the Ebro watershed, Spain and Sao Paulo state, Brazil

María Dolores Sendra[1,*], Viviane Moschini-Carlos[2], Marcelo L. M. Pompêo[3],
Xavier Soria[1], Juan Miguel Soria[1] and Eduardo Vicente[1]

[1] Instituto Cavanilles de Biodiversidad y Biología Evolutiva (ICBIBE). Universitat de València, 46980 Paterna, Spain.

[2] Departamento de Engenharia Ambiental, Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP), Instituto de Ciência e Tecnología, Campus de Sorocaba. Sorocaba-SP, Brasil.

[3] Departamento de Ecologia. Instituto de Biociências, Universidade de São Paulo (USP), Brasil.

[*] Corresponding author: sendrac@uv.es

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ABSTRACT

Ceratium furcoides (Levander) Langhans in reservoirs at the Ebro watershed, Spain, and Sao Paulo state, Brazil

This study compares the morphological characteristics of the dinoflagellate *Ceratium furcoides* from three Ebro basin reservoirs (Spain) and from six reservoirs at São Paulo state (Brazil) with the aim to identify the distinct morphotypes of *Ceratium furcoides* in Spain and Brazil. We studied the physical and chemical variables to determine the trophic state of each reservoir. Unlike *Ceratium hirundinella*, *Ceratium furcoides* is not a frequent species within the plankton community at Ebro Basin reservoirs, however, this species has been responsible of one monospecific bloom at El Val reservoir. Nevertheless, both *Ceratium* species are considered invasive in South America. *Ceratium furcoides* has displaced *C. hirundinella* at São Paulo state reservoirs, reaching high densities, to the extent of forming monospecific blooms. Morphometric analysis was performed by optic and electronic microscopy (DIC and SEM respectively). The analysis shows significant differences in: i) total cell length, ii) horns length and iii) presence or absence of a fourth horn. While the microscopic study allows differentiating morphotypes according to the reservoir typology, a deeper study using molecular genetic techniques on the different populations of *C. furcoides* would allow to know better the relationship between the dinoflagellate dynamics and the reservoirs trophic states.

Key words: *Ceratium furcoides*, morphotypes, reservoirs, algal blooms

RESUMEN

Ceratium furcoides *(Levander) Langhans en embalses de la cuenca del Ebro en España y embalses del estado de Sao Paulo en Brasil*

El presente estudio compara las características morfológicas del dinoflagelado Ceratium furcoides *procedente de tres embalses de la cuenca del río Ebro y de seis del estado de Sao Paulo con la finalidad de identificar los distintos morfotipos de* Ceratium furcoides *de España y Brasil. Se estudiaron las características físico-químicas para determinar el estado trófico de cada embalse. Contrariamente a* Ceratium hirundinella, Ceratium furcoides *es una especie poco común en la comunidad fitoplanctónica de los embalses de la cuenca del río Ebro, si bien en fechas recientes ha sido el responsable de un bloom monoespecífico en el embalse de El Val. Sin embargo, consideradas estas dos como especies invasoras en América del Sur,* Ceratium furcoides *ha desplazado a* Ceratium hirundinella *en los embalses del estado de Sao Paulo, alcanzando grandes densidades y llegando a formar en algunos casos blooms monoespecíficos. El análisis morfométrico, realizado mediante microscopia óptica y electrónica (DIC y SEM, respectivamente), muestra diferencias significativas en: i) la longitud total de la célula, ii) la longitud de los cuernos y iii) la presencia o no de un cuarto cuerno. Si bien el estudio microscópico permitió determinar diferentes morfotipos en función de la tipología de los embalses, un estudio utilizando técnicas de genética molecular en las diferentes poblaciones de* C. furcoides *permitiría una mejor comprensión de su dinámica en relación al estado trófico del embalse.*

Palabras clave: Ceratium furcoides*, morfotipos, embalses, blooms de algas*

INTRODUCTION

The *Ceratium* genera is known to occur in many temperate and subtropical areas of the world (Meichtry et al., 2016); it is a characteristic summer inhabitant at temperate stratified lakes with low epilimnetic nutrient concentrations at (Dokulil & Teubner 2003, Grigorszky et al., 2003 a, Grigorsky et al., 2003 b, Grigorsky et al., 2019). Inorganic nutrients are often cited as factors that trigger blooms of *Ceratium* (Whittington et al., 2000). According to Bustamante-Gil et al. (2012) *Ceratium hirundinella* (O. F. Muller) Dujardin and *Ceratium furcoides* (Levander) Langhans are ecologically similar.

Ceratium presents different morphologies associated with its life cycle, cyclomorphosis, and variations in certain environmental factors (Hickel, 1988; Lindstrom, 1992; Popovský & Pfiester, 1990; Bustamante Gil et al., 2012; Almanza et al., 2016).

In terms of morphology, *Ceratium furcoides* is a relatively large dinoflagellate with a body length between 162 and 322 µm, and a width between 28 and 42(56) µm (Popovský & Pfiester, 1990). Cells are fusiform, dorsoventrally flattened, with ventral area strongly concave. It has a narrowly conical epi-valve with a large horn and two, rarely three, horns in the hypo-valve (Hickel, 1988; Bustamante Gil et al., 2012). According to the literature, the epivalve of *Ceratium furcoides* is conical whereas in *Ceratium hirundinella* it is helmet-shaped. Nevertheless, the main difference between the two species is the apical plates disposition. *Ceratium hirundinella* has the four apical plates reaching the apex while in *Ceratium furcoides* only the first three apical plates (1′, 2′ y 3′) reach the apex and the fourth does not reach the apex and is shorter.

In South America the first record of *C. hirundinella* was made by Thomasson in 1963 from the Andean Patagonian lakes of Argentina. *C. hirundinella* began to colonize inland water bodies of Argentina, Chile, Bolivia, Brazil, in northward direction (Thomasson, 1963; Parra, 1998; Donagh et al., 2005; Ferrareze & Nogueria, 2006). But the invasion of *C. furcoides* is more recent (Santos-Wisniewski et al., 2007; Meitchtry et al., 2014) and appears to occur in the opposite direction; first through the colonization of those areas not occupied by the genus and subsequently invading environments inhabited by *C. hirundinella*, where was observed the species replacement in many cases (Meichtry et al., 2016).

Ceratium furcoides has been detected as invader in different aquatic ecosystems (Silva et al., 2012), showing advantages on resources competition against any native plankton species. (Sukenik et al., 2012) such as bigger size, selective predation from zooplankton community, better swim and migration in the water column, also, it can form blooms with high densities (Almanza et al., 2016; Cavalcante et al., 2016). In adverse environmental conditions *Ceratium furcoides*, like all the *Ceratium* species, is also cysts forming, being deposited in the sediment toremain in the aquatic ecosystem (Bellinger & Sigee, 2010; Reynolds, 2006). During the water mixing periods they can germinate (Cavalcante, et al., 2013) or remain encysted until the physical and chemical conditions of the water return to their optimum, thus being able to develop and form new blooms.

Ceratium species are tolerant to the stress, due their swim skills that allow vertical daytime migrations to several microhabitats where light and nutriments are more favorable (Cavalcante et al., 2013), besides they can tolerate different temperature and transparency conditions, which guarantees their ability to occur during all the year (Silva et al., 2012).

In addition to the resources expended for algal growth, at the decomposition layers a big amount of oxygen is consumed that it can reach anoxic conditions in the environment (Wetzel, 2001) leading the mortality of algae consumer fishes (Silva et al., 2012). If there are a high load of nutrients in the waterbody, this also favors the development of *C. furcoides* (Santos-Wisniewski et al., 2007; Matsumura-Tundisi et al., 2010; Bustamante-Gil et al., 2012), emphasizing that the eutrophication process can intensify blooms of this dinoflagellate (Almanza et al., 2016).

Although, *Ceratium* blooms are not considered to be toxic, it causes several impacts to continental aquatic ecosystems, affecting aesthetics and damaging the water supply system (Almanza et al., 2016), perhaps the better control of *C. furcoides* is the depletion of their resources (Santos-Wisniewski et al., 2007), generating competition between other phytoplankton organisms. Blooms of *C. furcoides* can be present in lakes and reservoirs with a trophic degree varying from mesotrophic to hypereutrophic (Bustamante-Gil et al., 2012; Silva et al., 2012; Meichtry et al., 2014).

Despite the blooms occurrence of this species does not present toxicity, this bio-invader characteristic has the capacity to perform other negative impacts to waterbody, such as: color and water taste alteration, obstruction of filters used in water treatment, besides, the damages to other organisms (Nicholls et al., 1980; Santos-Wisniewski et al., 2007; Silva et al., 2012; Meichtry et al., 2014).

In South America, *C. furcoides* has been recorded in the reservoir La Angostura (Bolivia) (Morales, 2016); also, in the lakes; Lobo (Argentina) (Bordet et al., 2017), Yacyretá (Argentina-Paraguay) (Meichtry et al., 2014), Río Grande II (Colombia) (Bustamante-Gil et al., 2012), and Lo Méndez (Chile) (Almanza et al., 2016). The dispersion and development in several Brazilian basins indicate that this species is in a process of expansion (Jati, et al., 2014). In Brazil the first record of the genera *Ceratium furcoides* was by Santos-Wisniewski et al. (2007) at the hydroelectric dam Furnas (Minas Gerais). In the last years, research in different areas of the country began to record it in some freshwater ecosystems (Silva et al., 2012; Meichtry et al., 2014), for example, at the state of Minas Gerais (Moreira et al., 2015), Billings (Matsumura-Tundisi et al., 2010), Guarapiranga (Nishimura, 2012, Nishimura et al., 2015), Jaguari, Jacareí (Hackbart et al., 2015) and Paiva Castro (Matta, 2016) reservoirs at São Paulo. At the high course of the Paraná river, Paraná st (Jati et al., 2014), Chopim, Capivari and São Jorge dams, at Rio Grande do Sul st, (Cavalcante et al., 2013), at the Itaúba dam (Cassol et al., 2014), Itá and Maia Filho reservoirs, Jacuí and Uruguai rivers (Cavalcante et al., 2013), Jacozinho and Toritama reservoirs in Pernambuco in the semiarid zones (Oliveira et al., 2016), Contas (Bahia) and Moxotó (Alagoas-Pernambuco) rivers, Sobradinho, Paulo Afonso (Bahia), Xingó (Alagoas-Sergipe) and Itaparica (Pernambuco-Bahia) reservoirs (Oliveira et al., 2011), Faxinal and Maestra reservoirs (Caixas do Sul, southern Brasil) (Cavalcante et al., 2016) besides, last records at pisciculture pools in São Carlos, São Paulost (Campanelli et al., 2016).

In Spain in general, contrary to *Ceratium hirundinella*, *Ceratium furcoides* is a not often specie. In the plankton community from the Ebro watershed, however, this specie has been the responsible of one monospecific bloom at El Val reservoir (Vicente et al., 2018), in the same way that in many Brazilian water bodies.

The main goal of this work is to compare the morphotypes of *C. furcoides* from Brazilian (southeast region) and Spanish (Ebro Basin) reservoirs with different trophic state with the aim to identify the distinct morphotypes of *Ceratium furcoides* in Spain and Brazil.

MATERIAL AND METHODS

Study Area

In Brazil, samplings were carried out during July 2015 at six reservoirs in São Paulo state: Atibainha (ATB), Barra Bonita (BB), Broa (BR), Igaratá (IGA), Itupararanga (ITUP) and Salto Grande (SG) (Fig. 1A). The Barra Bonita reservoir is the first of six waterbodies from a waterfall system of the Tietê River (Watanabe et al., 2016), together with the Salto Grande reservoir are in the most industrialized and populated area of the state (Rietzler et al., 2016). Itupararanga reservoir is located at the high course of the Sorocaba River that is the main tributary of Tietê River (Frascareli et al., 2015). Carlos Botelho (Lobo-Broa) is a eutrophic reservoir (maximum deep: 12 meters), located at Itirapina, (Tundisi et al., 2004; 2015), where agriculture is the permanent activity. The Atibainha reservoir, located near the

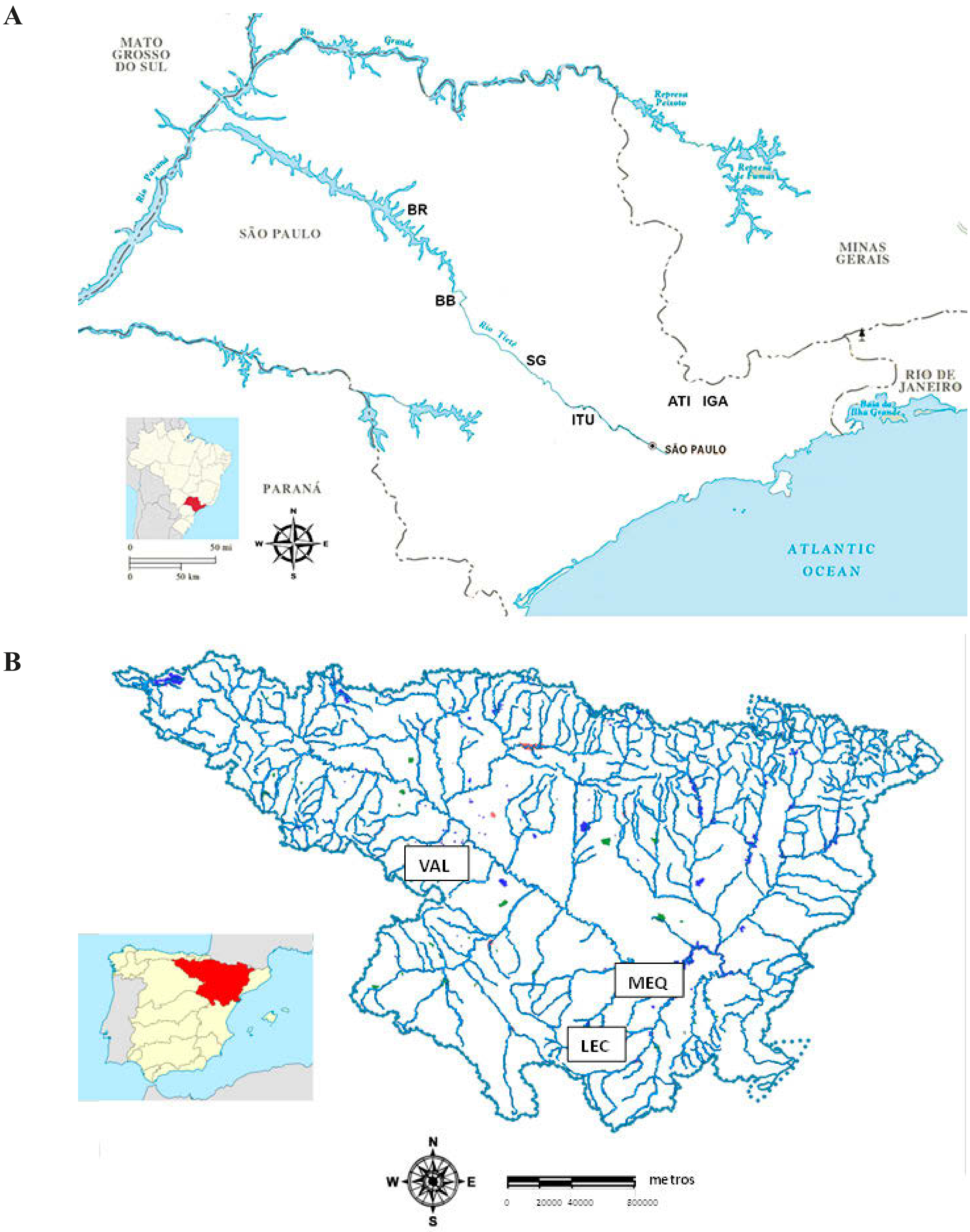

Figure 1. A) Location of the six studied reservoirs at São Paulo state, Brazil, identified by acronyms in the legend: BB: Barra Bonita, BR: Broa, SG: Salto Grande, ITU: Itupararanga, ATI: Atibainha. IGA: Igaratá. Source: Adapted from Frascarelli (2016). B) Location of the three studied reservoirs at the Ebro River watershed in Spain. LEC: Lechago, MEQ: Mequinenza and VAL: El Val. (Adapted from Ebro Basin Authority). *A) Localización de los seis embalses estudiados en el estado de São Paulo, Brasil, identificados por siglas en la leyenda: BB: Barra Bonita, BR: Broa, SG: Salto Grande, ITU: Itupararanga, ATI: Atibainha. IGA: Igaratá. Fuente: Adaptado de Frascarelli (2016). B) Localización espacial de los tres embalses estudiados en la cuenca del río Ebro, España. LEC: Lechago, MEQ: Mequinenza y VAL: El Val. (Adaptado de Confederación Hidrográfica del Ebro).*

city of Narazé Paulista, is part of the Cantareira System and is destined to drinking water supply for the metropolitan region of São Paulo (Carvalho, 2003). The Igaratá reservoir is located at Paraíba do Sul River basin near the city of Igaratá (state of São Paulo) (Frascareli, 2016).

In Spain, the samplings were carried out during July 2015 in three reservoirs of Ebro River watershed (northeast of Iberian Peninsula): Lechago (LEC), El Val (VAL) and Mequinenza (MEQ) (Fig. 1 B). Lechago reservoir is located at the eastern end of the Almazán basin, in the municipality of Calamocha, province of Teruel. The reservoir has a total capacity of 18.16 hm^3 and was built for water regulation of Pancrudo River. It is a monomictic reservoir, of calcareous geology, located in a non-humid zone belonging to headwaters and upper reaches section, with an average annual temperature less than 15 ºC. The Lechago reservoir is part of the Registry of Protected Areas elaborated by the Hydrographic Confederation of the Ebro, in response to Article 6 of Water Framework Directive, in the category of areas for water extraction for human consumption.

El Val reservoir is at Los Fayos municipality near Moncayo mountain range (province of Zaragoza). It is located on El Val River, tributary of the Ebro from the right bank. It regulates the waters of El Val River and receives some water contribution, arriving directly to the reservoir, by a pipeline from Queiles River. The reservoir has a total capacity of 25 hm^3. It is monomictic, calcareous, humid zone, belonging to riverheads and upper reaches section, with annual average temperature less than 15 ºC.

El Val reservoir is part of the Registry of Protected Areas prepared by the Hydrographic Confederation of the Ebro, in response to Article 6 of the Water Framework Directive, within the categories of areas of water extraction for human consumption and areas of habitats or species protection, ZEPA "Sierra de Moncayo - Los Fayos - Sierra de Armas (Natura 2000 Network, code: ES0000297).

Mequinenza reservoir dam is located at the municipality of Mequinenza, in the province of Zaragoza and regulates Ebro River waters. It is a large reservoir with a total capacity of 1534 hm^3. It is monomictic, calcareous geology, located in a non-humid zone and belonging to a low section of the Ebro main river axis. The Mequinenza reservoir is part of the Registry of Protected Areas prepared by the Hydrographic Confederation of the Ebro, in response to Article 6 of the Water Framework Directive, within the following categories: areas of water extraction for human consumption, sensitive areas under the framework of Directive 91/271 / EEC and habitat or species protection zones (Natura 2000 Network Point: SPA, ES0000182 "Valcuerna, Serreta Negra and Liberola") and its waters are mainly used for hydroelectric production and water supply to population.

In Spain, the reservoir sampling stations were stablished in the deepest part of each reservoir, at 300-500 m from the dam, during the summer months. A single integrated sample was taken in each reservoir, representative of the photic area, corresponding to water column until the depth arriving 1 % of surface light intensity (PAR). The integrated sample was carried out by means of a transparent reinforced wall plastic tube with inner light of 25 mm, weighted at one of its ends, which integrated the water column corresponds to the photic zone and whose content subsequently was mixed in a PET container. Then, from the integrated sample, different quantities of water were taken for in-situ processing, making additional measurements of physical, chemical and biological variables along the vertical profile by means of a 12 probes profiler (Sea-Bird 19 plus V2 (SBE19). After sampling, we proceed to water filtration for pigments extractions (Shoaf & Lium, 1976; Jeffrey & Humphrey, 1975) and laboratory analysis of other physical and chemical variables (Ammonium, total Phosphorus, and total Nitrogen) (Verdow et al., 1978; APHA, 1998; Ferree & Shannon, 2001) and phytoplankton variables (species identification, density and biovolume (MFIT-2013, version 1).

In Brazil the samples were collected at the river inflow zone, central and dam areas of each reservoir. A single integrated water column sample was collected, considering 2.7 times the depth of Secchi disc (m) (Cole, 1994). Water samples was analyzed at laboratory for the following variables: total phosphorus (Valderrama, 1981) and chlorophyll-*a* corrected for phaeophytin using 90 % acetone extraction (Lorenzen, 1967; Wetzel & Likens, 1991).

The Trophic state index (TSI) was determinate according to Lamparelli (2004) based in total phosphorus and chlorophyll-*a*. The limits used were: ultraoligotrophic (IET ≤ 47); oligotrophic (47 < IET ≤ 52); mesotrophic (52 < IET ≤ 59), eutrophic (59 < IET ≤ 63); supereutrophic (63 < IET ≤ 67) e hipereutrophic (IET > 67).

For phytoplankton analysis composition, as in the Spanish reservoirs, was used the methodology described in the Analysis Protocol of phytoplankton calculation and metrics in lakes and reservoirs (MFIT-2013, version 1) published by the Ministry of Agriculture, Food and Environment, Spain.

In both, Spain and Brazil, taxonomy and counting of algal individuals were made according Utermöhl method with a Nikon-ECLIPSE TE-2000S optical microscope and for fine morphological and morphometric characterization a Hitachi S-4800 electron microscope (SEM). The taxonomical analysis was based on Popovský and Pfiester (1990).

Significant difference between populations of *Ceratium furcoides* from the different reservoirs according to total length, width, apical horn length, antapical horn length, post-equatorial horn and fourth horn, were performed by statistical treatment (descriptive statistics, one-way ANOVA analysis ($P < 0.05$) and Tukey tests (Martínez-González et al., 2006)). Calculation of Pearson coefficient was used to find *Ceratium furcoides* relationships between different morphometric measurements in each reservoir (total length, width, apical horn length, antapical horn length, post-equatorial horn and fourth horn).

RESULTS

Water bodies located in the southeastern region of Sao Paulo state (Brazil) and the Ebro watershed in (Spain) presented different trophic states, from oligotrophic to hypereutrophic. The Igaratá, Atibainha, and Itupararanga reservoirs (Sao

Table 1. TSI of the different studied reservoirs along with the density and biovolume of *Ceratium furcoides* at each one of them. *TSI de los distintos embalses estudiados junto con la densidad y el biovolumen de* Ceratium furcoides *en cada uno de ellos.*

Reservoir	Sampling area	Total algae (Cel/ml)	*Ceratium* (Cel/ml)	*Ceratium* (%)	Total biovol (mm³/L)	*Ceratium* (mm³/L)	*Ceratium* (%)	TSI
Mequinenza	Dam Int 13 m	6867	1.20	0.02	0.78	0.13	16.60	Oligotrophic
Mequinenza	Dam 6 m	8240	2.00	0.03	1.20	0.26	21.70	Oligotrophic
Mequinenza	Dam 9 m	6525	2.00	0.04	1.07	0.26	24.40	Oligotrophic
Igarata	River	4592	467	10.17	56.98	52.47	92.07	Mesotrophic
Igarata	Center	5620	497	8.84	60.44	55.77	92.28	Mesotrophic
Igarata	Dam	4847	3241	66.86	365.70	363.83	99.49	Mesotrophic
Atibainha	River	198485	3209	1.62	392.61	360.31	91.77	Mesotrophic
Atibainha	Center	156078	2028	1.3	240.59	227.69	94.64	Mesotrophic
Atibainha	Dam	209157	1077	0.51	141.82	120.88	85.24	Mesotrophic
Lechago	Dam Int 7 m	25521	0.33	0.001	0.38	0.016	4.30	Mesotrophic
Itupararanga	River	464807	4903	1.05	673.72	550.44	81.70	Eutrophic
Itupararanga	Center	142837	366	0.26	106.03	41.08	38.74	Mesotrophic
Itupararanga	Dam	346307	146	0.04	71.43	16.43	23.00	Eutrophic
El Val	Dam Int 6 m	1588	382	24.1	43.54	43.44	99.80	Eutrophic
El Val	Dam 3 m	1140	842	73.9	95.75	95.73	99.98	Eutrophic
El Val	Dam 7 m	548	359	65.6	40.85	40.83	99.96	Eutrophic
Broa	River	1378414	94	0.007	263.58	10.56	4.01	Supereutrophic
Broa	Center	955358	0	0.000	345.91	0.00	0.00	Supereutrophic
Broa	Dam	599659	115	0.019	476.29	12.91	2.71	Supereutrophic
Barra Bonita	River	842075	7422	0.88	941.43	833.28	88.51	Hipereutrophic
Barra Bonita	Center	4998526	3146	0.06	969.33	353.26	36.44	Supereutrophic
Barra Bonita	Dam	3029142	648	0.02	523.98	72.77	13.89	Eutrophic
Salto Grande	River	1624999	355	0.02	266.28	39.90	14.99	Hipereutrophic
Salto Grande	Center	2072207	387	0.02	298.84	43.42	14.53	Supereutrophic
Salto Grande	Dam	146201	94	0.06	35.96	10.56	29.37	Supereutrophic

Paulo) and Lechago (Spain), were classified as mesotrophic, while Broa, Barra Bonita and Salto Grande reservoirs (Sao Paulo) and El Val reservoir (Spain) were the most eutrophics (supereutrophic to hypereutrophic) (Table1).

Morphotypes of *C. furcoides* in Brazilian and Spanish reservoirs showed differences in size, biovolume and number of horns (Fig. 2A, 2B). Organisms with three or four horns were observed in Mequinenza, Itupararanga and Barra Bonita, while in Igarata, Atibainha, Broa, El Val and Salto Grande the individuals do not present a fourth horn. However, only in Lechago all individuals present the fourth horn (Fig. 2A).

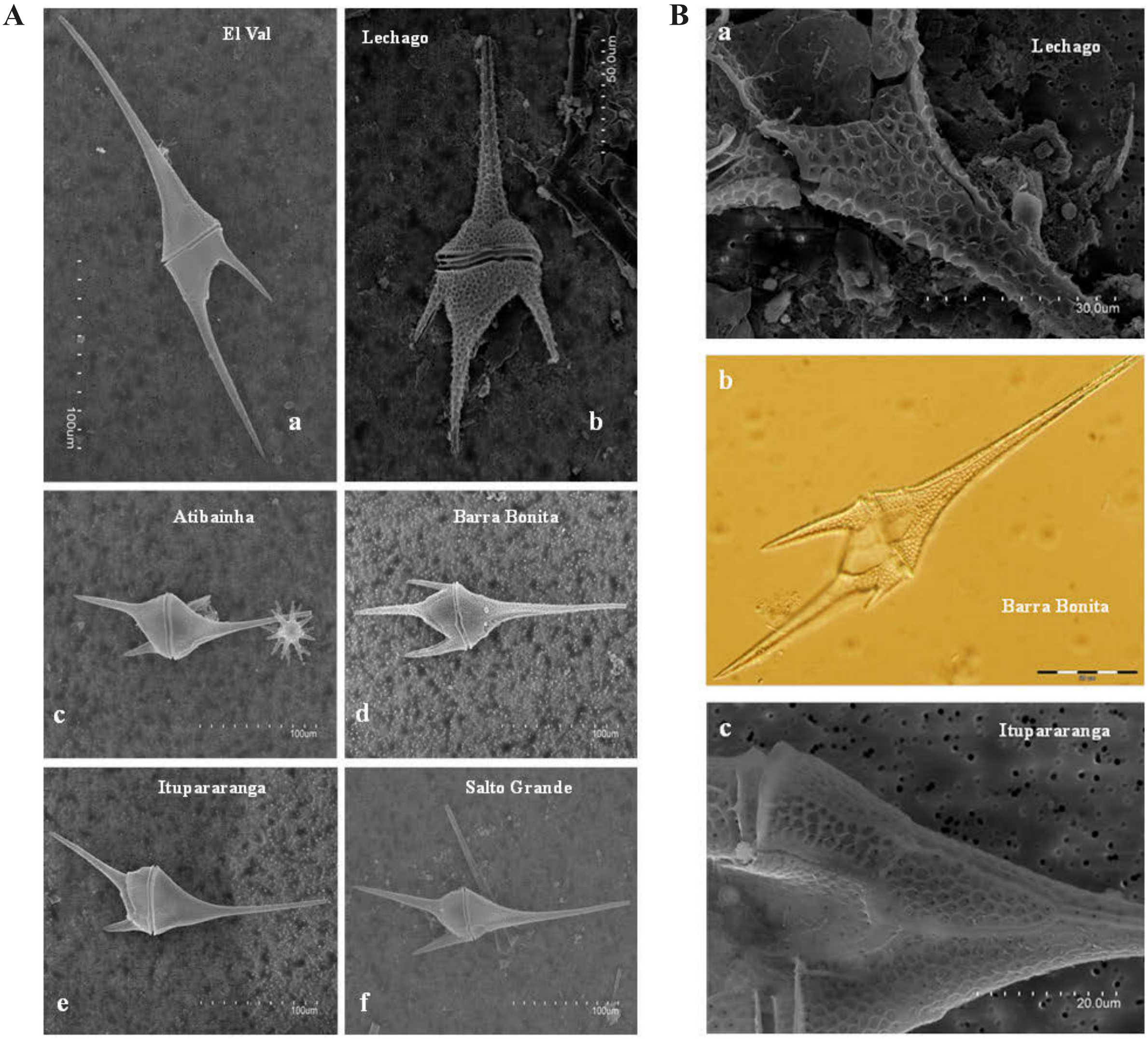

Figure 2. A) SEM images of *Ceratium furcoides* from several reservoirs where its morphological variability can be appreciated. a) El Val reservoir. b) Lechago reservoir. c) Atibainha reservoir. d) Barra Bonita reservoir. e) Itupararanga reservoir. f) Salto Grande reservoir. B). SEM images showing the characteristic plate 4' of *Ceratium furcoides* from the reservoirs: a) Lechago, b) Barra Bonita and c) Itupararanga. *A) Imágenes SEM de* Ceratium furcoides *de distintos embalses en las que se aprecia su variabilidad morfológica. a) Embalse de El Val. b) Embalse de Lechago. c) Embalse de Atibainha. d) Embalse de Barra Bonita. e) Embalse de Itupararanga. f) Embalse de Salto Grande. B) Imágenes SEM donde se puede apreciar la característica placa 4' de* Ceratium furcoides *de los embalses de: a) Lechago, b) Barra Bonita y c) Itupararanga.*

The morphometric study shows that the longer length individuals were present in El Val, with an average of 297 µm, followed by those in Barra Bonita (258 µm), whereas that the less length were in Lechago (185 µm) and Broa (156 µm) (Fig. 3a). The Tukey-test shows significative differences between the populations of El Val (Spain) and the rest of reservoirs, on the other hand the popula-

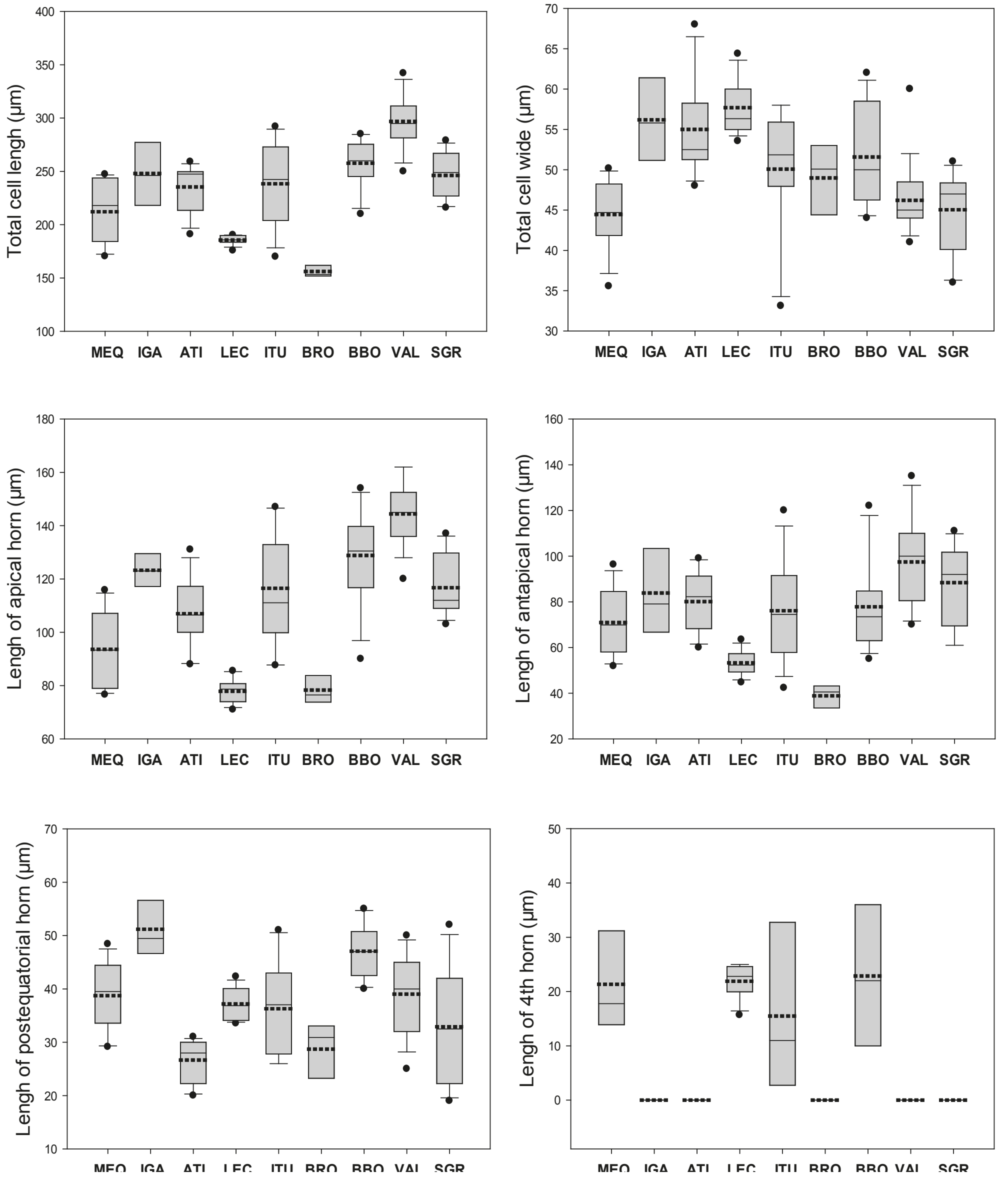

Figure 3. Morphometric measurements of *Ceratium furcoides* from the studied reservoirs (µm) with indication of the different morphotypes. a) Total length of the cell. Morphotype Types: morphotype 1 (M1), morphotype 2 (M2) morphotype 3 (M3) morphotype 4 (M4) and morphotype 5 (M5). b) Total width of the cell (cingulum). c) Length of the apical horn. d) Length of the antapical horn. e) Post-equatorial horn length. f) Length of the fourth horn. *Medidas morfométricas de* Ceratium furcoides *de los embalses estudiados (µm) con indicación de los distintos morfotipos. a) Longitud total de la célula. Morfotipos: morfotipo 1 (M1), morfotipo 2 (M2) morfotipo 3 (M3) morfotipo 4 (M4) y morfotipo 5 (M5). b) Ancho total de la célula (cíngulo). c) Longitud del cuerno apical. d) Longitud del cuerno antapical. e) Longitud del cuerno post-ecuatorial. f) Longitud del cuarto cuerno.*

Table 2. Significative morphometric differences among the measurements of *Ceratium furcoides* from the different reservoirs, according to the Tukey test. *Diferencias morfométricas significativas entre las medidas de* Ceratium furcoides *de los distintos embalses según la prueba de Tukey.*

Different populations (*) depending on Tukey's Test

	MEQ	IGA	ATI	LEC		BRO	BBO	VAL	SGR
MEQ	0					*		*	
IGA		0		*		*		*	
ATI			0			*		*	
LEC				0	*		*	*	*
ITU					0	*		*	
BRO						0	*	*	*
BBO							0	*	
VAL								0	*
SGR									0

According to total length (µm)

	MEQ	IGA	ATI	LEC	ITU	BRO	BBO	VAL	SGR
MEQ	0		*	*					
IGA		0							*
ATI			0					*	*
LEC				0					*
ITU					0				
BRO						0			
BBO							0		
VAL									
SGR									0

According to width (µm)

	MEQ	IGA	ATI	LEC	ITU	BRO	BBO	VAL	SGR
MEQ	0	*			*		*	*	
IGA		0		*		*		*	
ATI			0	*		*	*	*	
LEC				0	*		*	*	*
ITU					0	*		*	
BRO						0	*	*	*
BBO							0		
VAL								0	*
SGR									0

According to the AP horn (µm)

	MEQ	IGA	ATI	LEC	ITU	BRO	BBO	VAL	SGR
MEQ	0					*			
IGA		0				*			
ATI			0			*			
LEC				0				*	*
ITU					0	*			
BRO						0	*	*	*
BBO							0		
VAL								0	
SGR									0

According to the AN horn (µm)

	MEQ	IGA	ATI	LEC	ITU	BRO	BBO	VAL	SGR
MEQ	0		*						
IGA		0	*	*	*	*		*	*
ATI			0				*		
LEC				0					
ITU					0		*		
BRO						0	*		
BBO							0	*	*
VAL								0	
SGR									0

According to the PE horn (µm)

	MEQ	LEC	ITU	BBO
MEQ	0			
LEC		0		
ITU			0	
BBO				0

According to the 4th horn (µm)

tions of Lechago (Spain) and Broa (Brazil) do not have significant differences between them but are different against the rest of reservoirs (Table 2 and Fig. 3a). The rest of reservoirs: 5 from Brazil (Igarata, Atibainha, Salto Grande, Itupararanga and Barra Bonita) and 1 from Spain (Mequinenza) does not show any significant difference among their populations (Table 2 and Fig. 3a).

Regarding the individuals width (cingulum), the highest average was present in Lechago population with, 58 µm, and the lowest, width average 44.5 µm, at Mequinenza (Fig. 3b). Width mean measurement of *C. furcoides* populations presents the lowest significant differences among them (Table 2).

The major length of the apical horn was present in individuals from El Val, with an average of 144 µm, and the less length was in Lechago, average of 78 µm (Fig. 3c). The major length of the antapical horn as well was present in El Val (98 µm), nevertheless, the minor average length was in the individuals from Broa (39 µm) (Fig. 3d).

The average length of the post-equatorial horn varies from 27 µm (Atibainha) to 51 µm (Igaratá) (Fig. 3e). From all morphometric measurements is the length of the fourth horn which presents the major dispersion and do not shows significant difference between the populations (Table 2).

Correlation coefficients were performed with these different measurements and the major positive correlation was between the total length and length of the antapical horn, followed by the correlation of total length and the apical horn, except in Broa and Lechago reservoirs (Table 3) that in these waterbodies *Ceratium furcoides* shows the greatest correlation between the total length and the length of the apical horn (Table 3).

While measurement of the width (cingulum) is only significantly correlated in the reservoirs of Igaratá and Broa, in Itupararanga reservoir the correlation (r = 0.56) is with the apical horn. In Igaratá reservoir the significant correlation is with the apical horn (r = 0.87) as well as with the antapical (r = 0.92). Broa reservoir presents the highest correlation (r = 0.92) between the measurement of the cingulum and the length of the post-equatorial horn (Table 3).

The post-equatorial horn does not show any significant correlation among Mequinenza, Lechago and Barra Bonita reservoirs. However, El Val presents a positive correlation with the antapical horn. Igaratá and Atibainha reservoirs shows a positive correlation with the total length of the cell, while in Itupararanga, Broa and Salto Grande, beside their positive correlation with the total length, shows a correlation as well with the length of the antapical horn, cingulum length and length of the apical horn, respectively (Table 3).

In Lechago and Barra Bonita reservoirs the fourth horn does not show a positive significant

Table 3. Correlation coefficients (Pearson r) among the different *C. furcoides* morphometric measurements from each of the studied reservoirs. *Coeficientes de correlación (r de Pearson) entre las distintas medidas morfométricas de* C. furcoides *de cada uno de los embalses estudiados.*

Correlation Coefficients (r of Pearson) between the different measures in each reservoir

MEQ	L	B	AP	AN	PE	4ª
L	1.00					
B	0.20	1.00				
AP	**0.66**	-0.10	1.00			
AN	**0.89**	0.30	0.29	1.00		
PE	0.15	-0.19	-0.25	0.40	1.00	
4ª	**0.78**	-0.66	**0.59**	**0.52**	0.12	1.00

IGA	L	B	AP	AN	PE
L	1.00				
B	**0.93**	1.00			
AP	**0.86**	**0.87**	1.00		
AN	**0.95**	**0.92**	**0.90**	1.00	
PE	**0.56**	0.27	0.22	0.32	1.00

ATI	L	B	AP	AN	PE
L	1.00				
B	0.05	1.00			
AP	**0.77**	-0.27	1.00		
AN	**0.80**	0.11	0.42	1.00	
PE	**0.52**	0.33	0.20	0.42	1.00

LEC	L	B	AP	AN	PE	4ª
L	1.00					
B	0.39	1.00				
AP	**0.69**	0.18	1.00			
AN	0.18	-0.03	-0.28	1.00		
PE	0.46	0.41	0.22	0.29	1.00	
4ª	-0.04	0.13	-0.11	0.03	-0.30	1.00

ITU	L	B	AP	AN	PE	4ª
L	1.00					
B	**0.65**	1.00				
AP	**0.78**	**0.56**	1.00			
AN	**0.80**	0.43	0.33	1.00		
PE	**0.62**	0.31	0.46	**0.71**	1.00	
4ª	**0.69**	-0.52	0.38	0.07	**0.57**	1.00

BRO	L	B	AP	AN	PE
L	1.00				
B	0.41	1.00			
AP	**0.98**	0.31	1.00		
AN	**0.54**	0.22	0.42	1.00	
PE	**0.54**	**0.97**	0.45	0.40	1.00

BBO	L	B	AP	AN	PE	4ª
L	1.00					
B	0.32	1.00				
AP	0.30	-0.07	1.00			
AN	**0.51**	-0.34	0.20	1.00		
PE	0.13	-0.23	0.43	0.30	1.00	
4ª	-0.89	-0.18	-0.61	0.04	0.32	1.00

VAL	L	B	AP	AN	PE
L	1.00				
B	0.34	1.00			
AP	**0.68**	0.24	1.00		
AN	**0.74**	0.27	0.21	1.00	
PE	0.50	0.37	0.13	**0.63**	1.00

SGR	L	B	AP	AN	PE
L	1.00				
B	0.18	1.00			
AP	0.42	0.11	1.00		
AN	**0.65**	-0.37	-0.01	1.00	
PE	**0.75**	0.27	**0.76**	0.44	1.00

correlation with any other morphometric measurement, however in Mequinenza this correlation is present with the total length, apical and antapical horn length. Nevertheless, in Itupararanga the positive significant correlation of the fourth horn is with the total length and the post-equatorial horn length (Table 3).

Ceratium furcoides population from Lechago reservoir can be defined as a morphotype (M1), since all the organisms observed have four horns. This population has significant differences compared against the rest of reservoir populations, in addition to, presents the lowest variability among their individuals so, is the most homogenous population of the study.

The second morphotype (M2), can be the distinctive of El Val reservoir, where all the individual has three horns and the biggest size. These individuals are the most stylized, because they present the largest horns (apical and antapical), also, this population produce the only bloom observed in the Spanish reservoirs.

A third morphotype (M3) was found in the population of Broa, in this reservoir all the individuals have three horns and the great difference compared with the rest is their body size, since they are the smallest.

Finally, are two more morphotypes, the fourth (M4), where all the organisms present three horns (Igaratá, Atibainha and Salto Grande). The fifth (M5) with populations whose individuals have three or four horns, (Mequinenza, Itupararanga and Barra Bonita). The principal characteristic of this morphotype is the great dispersion of the length of the fourth horn compared against the other populations.

DISCUSSION

The morphological variability of *Ceratium furcoides* has been studied by important authors associating it mainly to several physico-chemical and environmental factors and to its life cycle during the seasonal succession at its water body (Almanza et al., 2016; Cavalcante et al., 2013; Lindström, 1992). In contrast, in the present work the morphological variability of *Ceratium furcoides* between several reservoirs has been studied at a given time of the succession.

Previous works have described how most individuals present two or three antapical horns depending on the advance of the succession, as in the case of Lake Lo Méndez (Almanza et al., 2016) or in the case of the reservoirs of Caxias do Sul (Cavalcante et al., 2013) or all individuals presented two antapical horns, as in Lake Erken (Lindström, 1992). In the different water bodies described at that time, individuals with two and three antapical horns coexisted or, on the other hand, all had two antapical horns. In our case, in the studied reservoirs all the possibilities are given, identifying three groups: 1) all the individuals presented three antapical horns, case of the Lechago reservoir, 2) individuals with two and three horns coexist, Mequinenza, Itupararanga and Barra Bonita and 3) all the individuals presented two antapical horns, Igarata, Atibainha, Broa, El Val and Salto Grande. Therefore, it is evident the high morphological variability of *Ceratium furcoides* and the need to deepen in the typical seasonal succession of each water body to be able to know if these groups remain along all the year and to associate this variability to the different physico-chemical and environmental conditions.

Ceratium abundance was associated with nutrient availability, especially phosphate and nitrate (Cavalcante et al., 2013). Similar results were found by Grigorszky et al. (2003), for dinoflagellates in Hungarian water bodies, and Cardoso et al. (2010), in subtropical reservoirs of Caxias do Sul.

In the urban lake Lo Mendez, Chile, the presence of *C. furcoides* was favored by the high nutrients concentration (Almanza et al., 2016) in concordance with previous research (Matsumura-Tundisi et al., 2010; Bustamante et al., 2012; Silva et al., 2012). In Fetsui Reservoir, Taiwan, the abundance of *Ceratium furcoides* was positively correlated with phosphorus, total organic carbon, bacterial numbers and the biochemical oxygen demand in the water (Wu & Chou, 1998). In Furnas reservoir, Brasil, Silva et al. (2012) related species abundance of *C. furcoides* with low temperatures and high nutrient concentrations (nitrate and nitrite) and in Colombia has been positively related to ammonium concentrations being considered a highly variable species at both temporal and spatial scales (Bustamante-Gil et al., 2012).

Reservoirs where high densities of *C. furcoides* were found in all mesotrophic, eutrophic or hypereutrophic conditions (Wu & Chou, 1998; Matsumura-Tundisi et al., 2010; Bustamante-Gil et al., 2012). Oliveira et al. (2016) also accepted the hypothesis that *C. furcoides* invasion and establishment in two reservoirs of the Capibaribe watershed was related to eutrophication. However, these studies differ from studies carried out by Ginkel et al. (2001), Koenig & Lira (2005) and Moreira et al. (2015) that indicated the presence of *C. furcoides* in ecosystems with low nutrients concentrations and also, the work of Donald et al. (2013) detected no significant influence of the ammonia and nitrate concentrations over the growth of *C. furcoides*. In our case, only one reservoir was classified as oligotrophic, Mequinenza, and the rest of the reservoirs studied were mesotrophic, eutrophic or hypereutrophic and even one of them was classified in its entirety as supereutrophic, the Broa reservoir, just the reservoir where the length of *C. furcoides* was the shortest of all those studied reservoirs.

The individuals of *C. furcoides* found in the samples measured 150.5-297 µm in length (L) and the width (W) varies from 33.1 µm to 68 µm. This morphometric and morphological measurements are consisting with the previous registers for this specie (Hansen & Flaim, 2007), Trentino Province, Italy, L: 165-260 µm, W: 30-50 µm; Oliveira et al. (2011), in semiarid region of Brasil, L: 194-228 µm, W: 43-60 µm; Pandeirada et al. (2013), Portugal, L: 255 µm, W: 44 µm; Almanza et al. (2016), Central Chile, L: 109.9-311.5 µm, W: 36.4-63.1 µm). The individuals described by Pandeirada et al. (2013) in Portugal by their measures and the stylized figure would remain our morphotype M2 together with the individuals described at El Val reservoir in Spain. Nevertheless, according to Nishimura et al. (2015) *C. furcoides* sampled at Billings and Guarapiranga reservoirs (São Paulo state, Brazil), presented a narrowly spindle-shaped, strongly dorsoventrally flattened, 114–154 µm long, 42–54 µm wide; epitheca formed into a narrow horn without shoulders; hypotheca broad and short, drawn out into two posterior horns of different lengths; plates smooth and with shallow net-like ornamentation. The length of those individuals is lower than the average length in all the reservoirs of the present study. On the other hand, the individuals found by Cavalcante et al. (2017) in the State of Paraná, Southern Brazil (L: 86–190 µm; W: 26–57.8 µm); Moreira et al. (2015), Southeast Brazil (L: 180-209 µm; W: 51-60 µm); Meichtry et al. (2014), Paraná river, Argentina-Paraguay (L: 146-212 µm; W: 37-45 µm); Jati et al. (2014), Upper Paraná, Brazil (L: 147.6-184.5 µm; W: 36.9-49.2 µm) and Cassol et al. (2014), Rio Grande do Sul, Brasil (L: 150-173 µm; W: 39-60 µm), would remain by their measurements between M1 and M3 morphotypes, the shortest ones. In absence of further data, the belonging of one or another morphotype would depend on the possession by all observed individuals of only three or four horns.

In the cases of very small and robust individuals, there would be a doubt and then, the same comment made by Cavalcante et al. (2017), they could be interpreted as *Ceratium rhomvoides* Hickel, a species closely related to *C. furcoides* (Hickel 1988) also described in Portugal by Pandeirada et al. (2013).

The contrasting behavior of *Ceratium* species in divergent lake types (with different climatic, morphometric, geological, hydrological, and trophic features) explains the existence of ecotypes of these species adapted to diverse environmental conditions and exhibiting high intra- and inter-population morphological variability (Salmaso, 2003). Bearing in mind the rapid dispersion of this species in South America and its capacity to form blooms with the negative consequences that this entails it is demonstrated the need to acquire a deeper knowledge of these species.

CONCLUSIONS

This study has constituted the first step identifying the distinct morphotypes of *Ceratium furcoides* in Spain and Brazil and corroborates the different morphologies associated with its life cycle and its relationship with physico-chemical and environmental factors.

Have been found significant differences in the morphometric analysis of the individuals of *C. furcoides* in some reservoirs: i) Total length; ii)

Length of the horns; iii) Presence or absence of the fourth horn, which have led to define the five different morphotypes.

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Zooplankton - phytoplankton relationships

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Can zooplankton grazing affect the functional features of phytoplankton in subtropical shallow lakes? - Experiment *in situ* in the south of Brazil

João Vitor Fonseca da Silva[1,*], Matheus Tenório Baumgartner[2], Maria Rosa Miracle[3,†], Juliana Déo Dias[4], Luzia Cleide Rodrigues[2] and Claudia Costa Bonecker[1,2]

[1] Programa de Pós Graduação em Biologia Comparada, Universidade Estadual de Maringá, Avenida Colombo, 5790, 87020-900, Maringá, PR, Brazil.
[2] Programa de Pós Graduação em Ecologia de Ambientes Aquáticos Continentais, Universidade Estadual de Maringá, Maringá, PR, Brazil.
[3] Departamento de Microbiología y Ecología, ICBiBE, Universidad de Valencia, Burjassot (Valencia), Spain.
[4] Departamento de Oceanografia e Limnologia, Universidade Federal do Rio Grande do Norte, Natal, RN, Brazil.

* Corresponding author: joaovitorbio@live.com

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ABSTRACT

Can zooplankton grazing affect the functional features of phytoplankton in subtropical shallow lakes? - Experiment *in situ* in the south of Brazil

Herbivory in aquatic environments reflects the organization of trophic webs as a structuring factor in the communities of primary producers. We experimentally evaluated the influence of grazing by zooplankton and by different predator groups on the abundance and biomass of phytoplankton and morphologically-based functional groups (MBFGs), in a subtropical floodplain shallow lake (Brazil). We tested if different grazing levels modify the structure of the phytoplankton, considering that higher zooplankton abundance reduces the abundance and biomass of phytoplankton and the MBFGs. We used five treatments (5 L) with different zooplankton abundance and five replicas to represent an increasing grazing gradient. Sampling of phytoplankton and zooplankton was carried out after 24 hours, in each treatment. The zooplankton abundance presented significant differences among treatments ($F = 11.79$; $p < 0.01$), showing the evident grazing gradient in the experiment. Rotifers dominated all the treatments, followed by copepods, although their abundance decreased at the end of the experiment. In contrast, cladoceran abundance increased. An inverse and significant relationship was observed between zooplankton and rotifer abundances and MBFG III abundance ($\beta = -0.85$, $p < 0.01$; $r^2 = 0.32$; $\beta = -0.54$; $p < 0.01$; $r^2 = 0.29$, respectively), showing an effective predator-prey interaction. Many positive and significant relationships were also observed among the grazers and the prey. The increase of the abundance and biovolume of phytoplankton was related to the bottom-up mechanisms, the rapid population growth of prey, and the short time of the experiment. The change in the MBFGs' contribution was related to the increase in the zooplankton and group abundance, grazer competition and exploitative competition among the MBFGs. The experiment contributes to the knowledge on an important part of the trophic dynamics among planktonic communities in subtropical floodplain shallow lakes.

Key words: experiment, predator-prey, morphologically-based functional groups, bottom-up mechanisms

RESUMO

O pastoreio de zooplâncton pode afetar as características funcionais do fitoplâncton em lagos rasos subtropicais? - Experimento in situ *no sul do Brasil*

Herbivoria em ambientes aquáticos reflete a organização de teias tróficas como fator estruturante das comunidades de produtores primários. Nós avaliamos a influência da predação do zooplâncton e dos diferentes grupos sobre a abundância e biomassa de fitoplâncton e grupos morfologicamente funcionais (MBFG), experimentalmente em um lago raso de uma planície de inundação *subtropical (Brasil). Testamos se diferentes níveis de predação modificam a estrutura do fitoplâncton, considerando que a maior abundância de zooplâncton reduz a abundância e a biomassa do fitoplâncton e dos MBFGs. Foram utilizados cinco tratamentos (5 L), com diferentes abundâncias de zooplâncton, e cinco réplicas, a fim de representar um gradiente crescente de predação. Amostras de fitoplâncton e zooplâncton foram obtidas após 24 horas, em cada tratamento. A abundância zooplanctônica mostrou diferenças significativas entre os tratamentos* ($F = 11.79$; $p < 0.01$), *evidenciando o gradiente de predação no experimento. Os rotíferos dominaram todos os tratamentos, seguidos pelos copépodes, embora sua abundância tenha diminuído ao final do experimento. Ao contrário, a abundância de cladóceros aumentou. Relação inversa e significativa foi observada entre abundância de zooplâncton e de rotíferos e a abundância de MBFG III* ($\beta = -0.85$, $p < 0.01$; $r^2 = 0.32$; $\beta = -0.54$; $p < 0.01$; $r^2 = 0.29$, *respectivamente), mostrando uma eficiente relação predador-presa. Relações positivas e significativas também foram observadas entre os predadores e as presas. O aumento da abundância e do biovolume do fitoplâncton foi relacionado aos mecanismos bottom-up, ao rápido crescimento populacional das presas e o curto tempo do experimento. A mudança da contribuição dos MBFGs foi relacionada ao aumento da abundância do zooplâncton e dos grupos, competição entre predadores, e competição exploratória entre os MBFGs. O experimento contribuiu para o conhecimento de uma parte importante da dinâmica trófica entre comunidades planctônicas em lagos rasos de planície de inundação subtropical.*

Palavras chave: *experimento, predador-presa, grupos morfologicamente funcionais, mecanismos bottom-up*

INTRODUCTION

Zooplanktonic organisms occupy specific niches, and changes in the dominance pattern of these grazers affect prey populations according to the diet complexity of zooplankton species (DeMott, 1986). In addition, the prey selectivity, which regulates the exploitative competition among phytoplankton species (DeMott, 1989), can also facilitate the uncontrolled growth of undesired populations, such as cyanobacteria, because grazing acts preferably on the more natural competitors (Leitão *et al.*, 2018).

The effect of zooplankton grazing on phytoplankton depends on the composition, density, and body size of grazers (Cyr & Curtis, 1999; Lacerot *et al.*, 2013). In general, small-sized zooplankton (rotifers, small cladocerans, and nauplii) groups feed on small organisms (Cyr & Curtis, 1999), while large-sized groups vary their feeding strategy as filter feeders (calanoid copepods and clacocerans), omnivores (cyclopoid copepods and cladocerns) and carnivores (cyclopoid copepods) (Declerck *et al.*, 2007; Özkan *et al.*, 2014; Tõnno *et al.*, 2016). Thus, it can be considered that aquatic trophic webs are strongly influenced by the groups or species that are available for consumption (Modenutti *et al.*, 2003; Qiu *et al.*, 2016). Studies revealed that zooplankton is responsible for consuming most of the phytoplankton productivity that is transferred to other trophic levels (Pfister & Arndt, 1998; Yang *et al.*, 2016). Along with feeding preference and selectivity by zooplankton, competition among grazers also affects the structure of prey communities, because changes in the relative abundance among grazers can result in potential different grazing levels (Brooks & Dodson, 1965).

Therefore, the interaction between zooplankton and phytoplankton controls ecosystem processes such as productivity, energy flow, and nutrient cycling (Quintana *et al.*, 2015). The particular interactions among different species of prey and predators can result in the consumption of different functional groups of prey, with potential effects on ecosystem processes and services. Specifically, rotifers are potential grazers of small organisms (up to 5 μm) with high surface/volume ratio and low nutritional value (Brandl, 2005, Kruk *et al.*, 2010) and, as along with cladocerans, they may graze on medium-sized organisms without specializations represented by many classes (Chlorophyceae, Xanthophyceae, and Zygnemaphyceae) (Colina *et al.*, 2016). Copepods preferentially graze on medium to large-sized unicellular flagellates (up to 11.6 μm) with high nutritional value, and on organisms with low mortality, silicate exoskeleton, and high-density cells (Kruk *et al.*, 2010; Colina *et al.*, 2016).

The lakes of the upper Paraná River floodplain present a great diversity of zooplankton and phytoplankton species, and high trophic complexity (Roberto *et al.*, 2009; Agostinho *et al.*, 2009;

Lansac-Tôha *et al.*, 2009; Bortolini *et al.*, 2016). However, the structure of trophic interactions in these ecosystems can provide important insights about the drivers of spatial and temporal changes in productivity and energy flow. Therefore, our objective was to evaluate the influence of different grazing levels by zooplankton on the phytoplankton community in floodplain lakes. Specifically, we experimentally assessed how the gradient of abundance of zooplankton and groups (rotifer, cladoceran and copepod) affects the abundance and biovolume of phytoplankton and morphologically-based functional groups (MBFGs) in a subtropical floodplain shallow lake, in the South of Brazil. We tested the hypothesis that higher zooplankton abundance reduces the abundance and biomass of phytoplankton and MBFGs (MBFG I-MBFG VII). Specifically, the response of phytoplankton to grazing is likely to depend on the prevailing group of grazers due to feeding preference and selectivity. The investigations of these biotic relationships between planktonic communities contributes to understanding the trophic dynamics of river-floodplain systems, particularly about the basis of food webs.

MATERIAL AND METHODS

Experimental Design

The experiment was conducted *in situ*, in Garças Lake (22º 43' 27.18" S and 53º 13' 4.56" W), which is located in the upper Paraná River floodplain. This lake is 2128 m in length, with an area of 14 ha and a perimeter of 4338 m. This environment is directly under the influence of water-level variations of the Paraná River, since it is permanently connected to this river.

We conducted the experiment on December 10, 2016 (summer), during a period of 24 hours. The environmental conditions of the lake at the beginning of the experiment were observed: depth (1.60 m), water temperature (27 ºC), pH (7.36), dissolved oxygen (2.02 mg/L), electric conductance (71.80 μS/cm), total nitrogen (74.29 μg/L), total phosphorous (46.90 μg/L), nitrate (14.88 μg/L), and phosphate (6.48 μg/L).

The study design involved the manipulation of the abundance of zooplankton, simulating an increasing grazing gradient, considering that the effect of grazing on phytoplankton depends on the community structure of grazers. We used a dilution/concentration technique, which is employed to control the grazing of the zooplankton on primary producers (Landry & Hassett, 1982; Calbet *et al.*, 2012), and is based on actively manipulating the abundance of zooplankton through sequential dilutions and concentrations of the lake water. This procedure indirectly controls the encounter rate between grazer and prey.

Each sampling unit was composed of a plastic bottle (5 L), attached to a floater and randomly placed in the lake. This floating structure allowed bottles to stay suspended in the sub-surface, without shading. We had five replicates (bottles) of each of the five treatments representing the grazing gradient (see description below), totalizing 25 sampling units (microcosms).

For the preparation of mesocosms, water was previously collected at many sites within the lake using a water pump, and it was then mixed and stored in a water tank (500 L) inside the boat. This water represented the observed zooplankton-phytoplankton relationship of the lake and was used in all treatments to ensure the homogeneity of the initial experimental conditions. The dilutions of predators were performed with the water from the tank, and filtered with a planktonic net (10 μm) to retain zooplankton. To avoid unpredicted changes in the phytoplankton structure due to a shortage of resources and potential community collapse, we supplied each sampling unit with low-concentration nutrients in the mesocosms (10 μg/L of nitrate and 0.5 μg/L of phosphate, considering N:P = 20:1).

Samples of phytoplankton and zooplankton obtained from the lake were considered as the communities at the beginning of the experiment. The phytoplankton was collected in 100 mL of water, fixed with acetic Lugol. The zooplankton was collected from 5 L of water filtered in a plankton net (45 μm), fixed with formalin 40 % buffered with calcium carbonate.

We simulated five different scenarios of the predator-prey relationship; manipulating the zooplankton density through dilution and concentration (the filtration with the 45-μm plankton net retained all zooplankton). Therefore, treatments represented: (i) dilution of the abundance of grazers to ¼ of that observed in the lake, or 25 % of grazers (T1), containing 1.25 L directly from the tank and 3.75 L of filtered water; (ii) dilution of the abundance of grazers to ½ of that observed in the lake, or 50 % of grazers (T2), containing 2.5 L directly from the tank and 2.5 L of filtered water; (iii) abundance of grazers observed in the lake (T3), with 5 L directly from the tank, without dilution or concentration; (iv) 2x the abundance of grazers in the lake (T4), containing 10 L from the tank, concentrated into 5 L using the plankton net (45 µm); and (v) 4x the abundance of grazers in the lake (T5), containing 20 L from the tank, concentrated into 5 L using the plankton net (45 µm).

Experimental units were exposed for 24 hours, and then we took samples of 100 mL of each unit, fixed with acetic Lugol, to analyze phytoplankton qualitatively and quantitatively, and filtered 4.35 L of each sampling unit in a plankton net (45 µm), fixed with formalin 40 %, to analyze zooplankton. The remaining water of each experimental unit was used to calculate the biovolume.

Analysis of Communities

The quantitative analysis of the phytoplankton community was performed according to Utermöhl (1958), in an inverted microscope (Carl Zeiss) with a millimetric ocular attached. Counting was carried out in random fields, until the stabilization of the increment of individuals per class, and the result was expressed as individuals (cells, colonies or filaments) per mL. The classification of Cyanobacteria followed Komárek & Anagnostidis (1986, 1989, 1998, 2005), and other groups followed Reviers (2003). Identified taxa were further classified into seven morphological-functional groups (MBFG) following Kruk *et al.* (2010), according to the characteristics of each taxon (e. g. presence of aerotopes, flagella, mucilage, heterocyst, presence of silica, and volume). The biomass of phytoplankton (mm^3/L was estimated from the individual biovolume, considering the geometric forms of algal cells, following Hillebrand *et al.* (1999), Sun & Liu (2003) and Fonseca *et al.* (2014). We took measurements of volume, surface, maximum linear dimension, width, and height for each taxon, for a minimum of 50 individuals, or from all individuals when abundance was low.

The identification of the zooplankton species was performed in an optic microscope (Olympus CX41), following specialized literature (Smirnov, 1971, 1976, 1992; Koste, 1978; Elmoor-Loureiro, 1997; Dussart, 1984; Reid 1985). For the information on zooplankton community composition recorded in the experiment (See Table S1, is available at http://www.limnetica.net/en/limnetica). The abundance was estimated by counting at least 50 individuals of each group (rotifers, cladocerans, and copepods), in three sequential sub-samplings (Bottrell *et al.*, 1976), obtained with a Stempell pipette (2.5 mL), in a Sedgwick-Rafter chamber. Samples with a low number of individuals were quantified entirely and results were expressed as ind/L.

Zooplankton biomass was estimated from the biovolume of rotifers (Ruttner-Kolisco, 1977), the length–weight relationships of microcrustaceans reported for the floodplain (Azevedo *et al.*, 2012), and literature, mostly from tropical regions (Dumont *et al.*, 1975; Melão, 1999; Maia-Barbosa & Bozelli, 2005). Measurements (length, width, and height) of up to 30 individuals of each species of rotifers, cladocerans, and adult and juvenile copepods, were taken in each sample.

Data analysis

A one-way Analysis of Variance (ANOVA) was used to test for differences in the abundance of zooplankton (grazers) among treatments (T1 to T5), and thus ensure the efficiency of the experimental design. Data on zooplankton abundances had been transformed previously (log (x+1)).

Simple linear regressions using ordinary least squares (Zar, 2009) were used to evaluate the effect of the grazing gradient of the zooplankton (total abundance and abundance of different groups – rotifers, cladocerans, and copepods) on phytoplankton (total abundance and biovolume from different MBFGs). Data used in regressions were those from the results of each sampling unit, having subtracted the results from lake samples (referential conditions) of predator and prey. These data were transformed (log10(x+1)) to approximate relationships to linear. Regression models were fitted using function “lm” from the package “stats” in the R Environment (R Core Team, 2013). The assumptions of residual normality and homoscedasticity were tested using Shapiro-Wilk and Levene’s test, respectively. Analyses were considered significant at the level of $\alpha = 0.05$ and developed using the package “vegan” (Oksanen *et al.*, 2013). Graphs were built in Statistica 7.0 (Statsoft Inc., 2005).

RESULTS

Phytoplankton and Zooplankton

The phytoplankton abundance was mainly represented by Chlorophyceae, followed by Cryptophyceae and Cyanobacteria in all treatments, and Zygnemaphyceae in the lake. Considering the biovolume of algae, Cyanobacteria dominated in all experiments, followed by Chlorophyceae, and Bacillariophyceae dominated in the lake (Figs. 1a and 1b).

The total abundance and especially the biovolume of phytoplankton increased along the grazing gradient and presented very high values when compared to the lake (Figs. 1a and 1b). It was also noted that the contribution of the functional groups varied. MBFG VII had higher biovolume in most of the treatments (T3, T4, and T5) (Fig. 2a) and MBFG V had higher abundance in all treatments (Fig. 2b).

As expected from the experimental design, the abundance of zooplankton presented significant variation along the grazing gradient (ANOVA, $F_{(4,\,2.01)} = 16.87$; $p < 0.01$). The post-hoc Tukey test revealed that the average abundance of zooplankton in treatment T1 was significantly different from T4 and T5, and that T3 was different from T5 ($p < 0.05$) (Fig. 3a). We confirmed the efficiency of the dilution/concentration procedure, because the zooplankton abundance was lower in the treatment with more dilution of the predators (T1) and higher in treatments where predators were concentrated (T4 and T5). Rotifers were numerically dominant in all treatments and in the lake, followed by copepods and cladocerans (Figs. 3a and 3b).

Comparing the beginning (lake) and end of the experiment (treatments), similar values

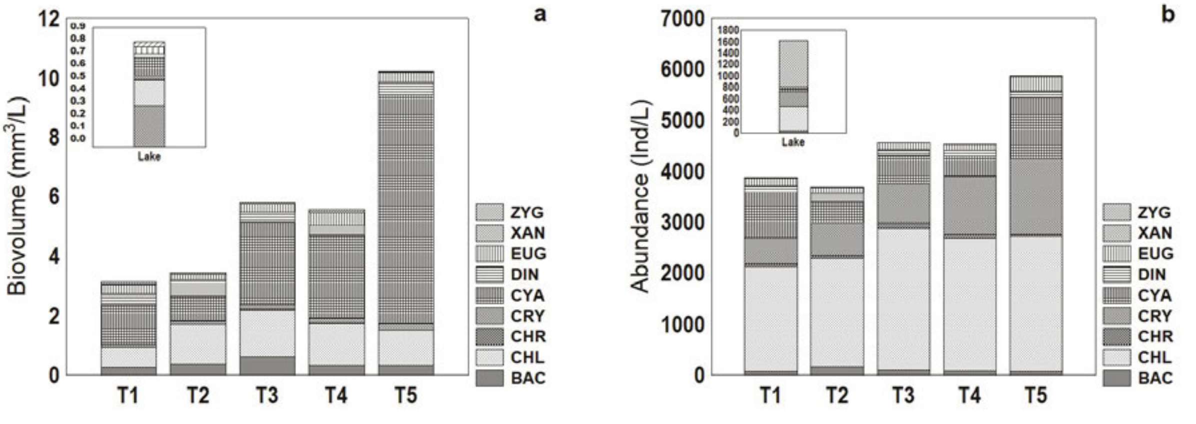

Figure 1. Total (a) abundance (ind/L) and (b) biovolume (mm^3/L) of phytoplankton classes observed in the lake (beginning) and in different treatments (end) from the grazing experiment conducted in Garças Lake, in the Upper Paraná river floodplain (Brazil). ZYG = ZYGNEMAPHYCEAE; XAN = XANTHOPHYCEAE; EUG = EUGLENOPHYCEAE; DIN = DINOPHYCEAE; CYA = CYANOBACTERIA; CRY = CRYPTOPHYCEAE; CHR = CHRYSOPHYCEAE; CHL = CHLOROPHYCEAE; BAC = BACILLARIOPHYCEAE. *Abundância total (ind/L) (a) e biovolume (mm^3/L) (b) das classes do fitoplâncton observadas na lagoa (início) e em diferentes tratamentos (final) do experimento de predação, realizado na Lagoa das Garças, na planície de inundação do alto rio Paraná (Brasil). ZYG = ZYGNEMAPHYCEAE; XAN = XANTHOPHYCEAE; EUG = EUGLENOPHYCEAE; DIN = DINOPHYCEAE; CYA = CYANOBACTERIA; CRY = CRYPTOPHYCEAE; CHR = CHRYSOPHYCEAE; CHL = CHLOROPHYCEAE; BAC = BACILLARIOPHYCEAE.*

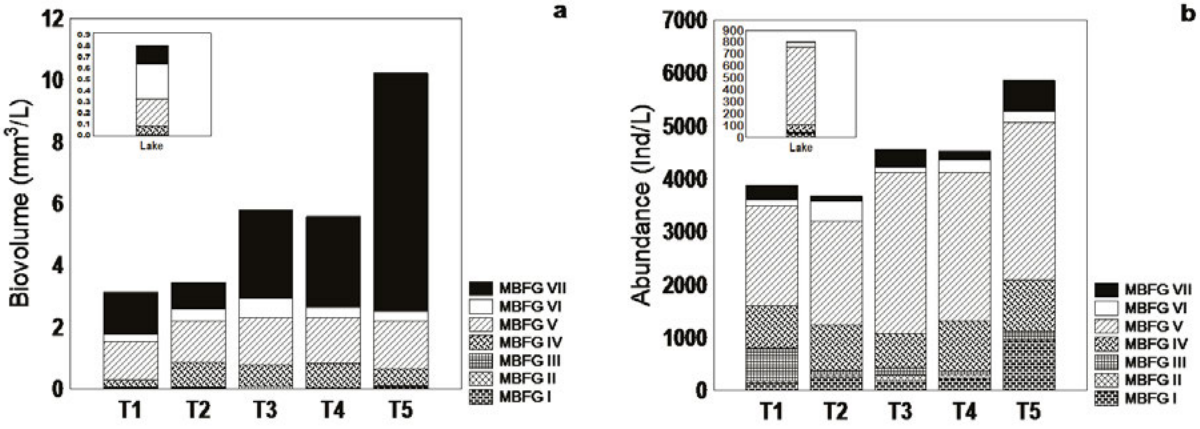

Figure 2. Total and morphologically-based functional groups (MBFG) phytoplankton biovolume (mm^3/L) (a) and abundance (ind/L) (b), observed in the lake (beginning) and in different treatments (end) from the grazing experiment conducted in Garças Lake, in the Upper Paraná river floodplain (Brazil). *Biovolume total e dos principais grupos morfológicos funcionais (MBFG) do fitoplâncton (mm^3/L) (a), e abundância (ind/L) (b), observados na lagoa (início) e em diferentes tratamentos (final) do experimento de predação, realizado na Lagoa Garças, na planície de inundação do alto rio Paraná (Brasil).*

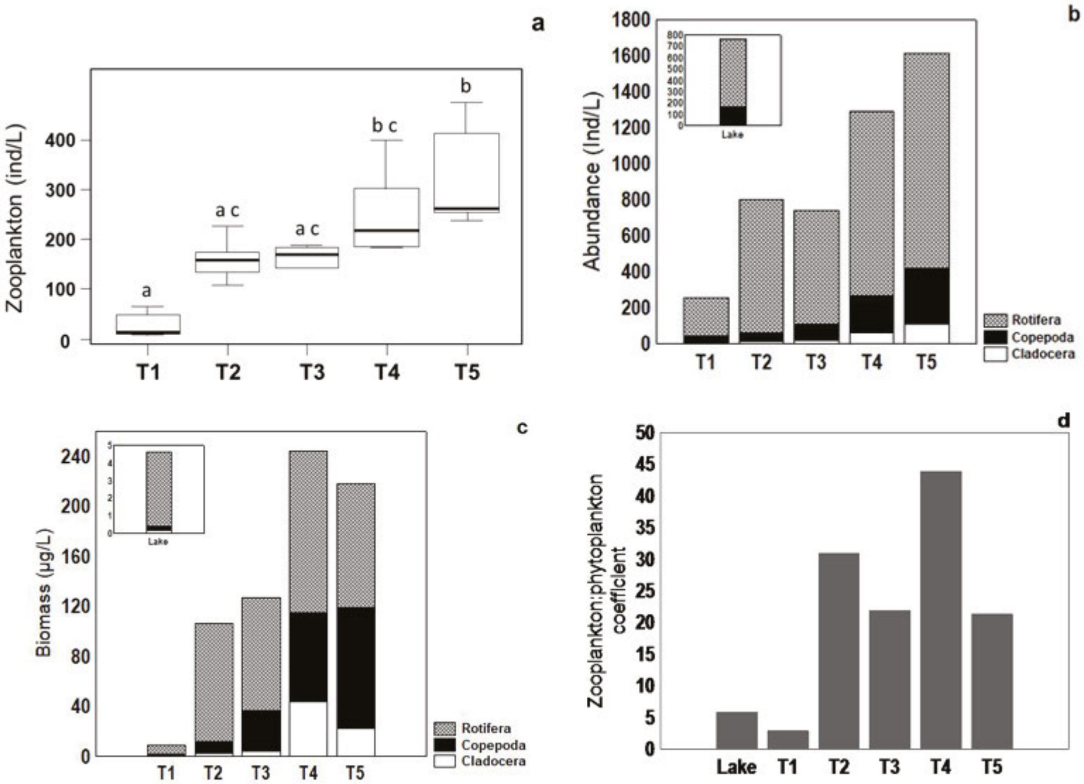

Figure 3. Zooplankton abundance (ind/L) (a) (median (central bar), quartiles (boxes), and range (whiskers), abundance (ind/L) (b) and biomass (µg/L) of the zooplankton groups (c), and zooplankton:phytoplankton coefficient (d) registered in the lake (beginning) and in different treatments (end) from the grazing experiment conducted in Garças Lake, in the Upper Paraná river floodplain (Brazil). Letters in a represent the results of the Tukey test. *Abundância do zooplâncton (ind/L) (a) (mediana (barra central), quartis (caixas) e amplitude (whiskers)), abundância (ind/L) (b) e biomassa (c) dos principais grupos zooplanctônicos, e zooplâncton:fitoplâncton coeficiente (d) registradas na lagoa (início) e nos diferentes tratamentos (final) do experimento de predação, realizado na Lagoa Garças, na planície de inundação do alto rio Paraná (Brasil). As letras em a representam os resultados do teste de Tukey.*

were observed in the lake and the treatment with less dilution (T2) and the treatment without manipulating grazers (T3) (Fig. 3b). The higher abundance observed in concentered treatments should already be higher at the beginning. However, the proportion of community abundance between the beginning and the end of the experiment was not maintained. The abundance of rotifers and copepods decreased, and the abundance of cladocerans increased at the end of the experiment, although rotifers continued to dominate.

The zooplankton biomass, as expected, was greater in the concentrated treatments (T4 and T5). Rotifers also showed the highest values in all treatments, cladocerans in T4 and copepods in T5 (Fig. 3c).

Responses of Phytoplankton to the Grazing Pressure by Zooplankton

The gradient of grazing pressure by zooplankton influenced MBFG III (large filaments with aerotopes) because the relationships between the abundance of zooplankton and rotifers and the biovolume of these algae were only significant and inversely related ($\beta = -0.85$, $p < 0.01$; $r^2 = 0.32$; $\beta = -0.54$; $p < 0.01$; $r^2 = 0.29$, respectively) (Table 1).

In contrast, positive and significant relationships were found between (i) the zooplankton abundance and the biovolume of phytoplankton and MBFG IV and MBFG VII; (ii) the rotifer abundance and the biovolume of phytoplankton and MBFG VII; (iii) the cladoceran abundance

Table 1. Linear regression models between the abundance of predators (zooplankton) and features of the prey (phytoplankton) community, from the grazing experiment conducted in Garças Lake, upper Paraná River floodplain (Brazil). R^2 is the coefficient of determination, β_0 and β_1 are the intercept and slope coefficient from linear models, respectively, and *p* is the significance at $\alpha = 0.05$. *Modelos de regressão linear entre a abundância de predadores (zooplâncton) e características da comunidade de presas (fitoplâncton), a partir do experimento de predação realizado na Lagoa Garças, na planície de inundação do alto rio Paraná (Brasil). R^2 é o coeficiente de determinação, β_0 e β_1 são o intercepto e o coeficiente de inclinação dos modelos lineares, respectivamente, e* p *é a significância em $\alpha = 0.05$.*

Predator	Prey	R^2	β_0	β_1	*p*
Zooplankton (ind/L)	Total biovolume (mm^3/L)	0.248	-0.124	0.184	0.011
	MBFG IV biovolume (mm^3/L)	0.166	-0.030	0.032	0.043
	MBFG VII biovolume (mm^3/L)	0.246	-0.215	0.180	0.011
	MBFG III biovolume (ind/L)	0.321	3.054	-0.848	0.003
Rotifers (ind/L)	Total biovolume (mm^3/L)	0.172	0.067	0.103	0.030
	MBFG VII biovolume (mm^3/L)	0.179	-0.032	0.103	0.030
	MBFG III abundance (ind/L)	0.291	2.302	-0.540	0.005
Cladocerans (ind/L)	Total Biovolume (mm^3/L)	0.448	0.046	0.276	0.001
	MBFG I biovolume (mm^3/L)	0.324	-0.001	0.004	0.002
	MBFG V biovolume (mm^3/L)	0.288	0.045	0.055	0.005
	MBFG VII biovolume (mm^3/L)	0.389	-0.034	0.253	0.001
	Total Abundance (ind/L)	0.379	2.434	0.456	0.001
	MBFG V abundance (ind/L)	0.241	2.558	0.162	0.01
Copepods (ind/L)	Total Biovolume (mm^3/L)	0.438	-0.117	0.291	0.001
	MBFG I biovolume (mm^3/L)	0.364	-0.003	0.004	0.001
	MBFG V biovolume (mm^3/L)	0.379	-0.005	0.067	0.001
	MBFG VII biovolume (mm^3/L)	0.387	-0.187	0.269	0.001
	Total Abundance (ind/L)	0.345	2.187	0.463	0.002
	MBFG V abundance (mm^3/L)	0.265	2.449	0.180	0.008

and the biovolume of phytoplankton and MBFG I, MBFG V and MBFG VII; (iv) the phytoplankton abundance and MBFG V; and (v) the copepod abundance and the biovolume of phytoplankton and MBFG I, MBFG V and MBFG VII, as well as with the abundance of phytoplankton and MBFG V (Table 1). The zooplankton:phytoplankton coefficient (Jeppesen *et al.*, 2003) was highest in T2 and T4 (Fig. 3d), where MBFG V dominated in the phytoplankton biovolume and abundance, and MBFG VII in the biovolume (Figs. 2a and 2b). More information recorded in the experiment is provided in Fig. S1 (Supplementary Material is available at http://www.limnetica.net/en/limnetica).

DISCUSSION

The grazing pressure gradient did not affect the phytoplankton abundance and biovolume due to the increase of these attributes in all treatments when compared to the lake. This finding was expected for the dilution treatments but not for the concentrated treatments. The MBFG attributes also increased in the treatments, but their contribution changed. For instance, a greater biovolume of MBFG VI was observed in the lake than in the treatments, and the contribution of MBFG V abundance did not change between the lake and treatments. Therefore, it was not possible to confirm the existence of grazing pressure among the grazers and the prey.

The establishment of the predator-prey relationship depends on the different types of predator in the environment, and also on the feeding selectivity of these organisms and their ability to select, manipulate, and consume prey (Lampert *et al.*,1986; Hansen *et al.*, 1994). In this context, the inverse relationships between the abundances of zooplankton, rotifers and MBFG III was addressed to the high abundances of small algae (e. g. *Pseudoanabena mucicola* with 7.5 µm x 2.5 µm). Thus, small algae should be preferential prey for rotifers, which dominated in the grazer community. The aerotopes, which facilitate floating and make them more accessible as prey in the water column, could have favored grazing (Walsby, 1994). Thus, among the zooplankton, rotifers clearly represented grazing pressure.

In contrast, the many positive and significant relationships observed between the abundance of zooplankton, rotifers, cladocerans and copepods, and the abundance, biovolume, and morphologically-based functional groups of phytoplankton showed a weak predator-prey relationship, which allowed the grazer populations to be maintained at the end of the experiment. These relationships also suggested the direct effect of nutrient release (bottom-up mechanism) overlapping with the predator-prey relationship (top-down mechanism) (Carpenter *et al.*, 1985). The nutrients supplied by the disruption of the algal cells (sloppy feeding) and/or the excretion of nutrients by zooplankton, may have favored the growth of phytoplankton, during the experiment (Oliver *et al.*, 2014). Considering the synergic role of these two mechanisms, some studies state that positive correlations between predator and prey abundances occur when the prey growth rate exceeds the grazing rate, and the growth is limited only by the availability of resources (Pereira *et al.*, 2005). Moreover, the growth of the prey populations is faster than those of the predator.

In our study, the increase in the abundance and biovolume of the morphologically-based functional groups is also supposed to be related to the low efficiency of grazing due to the short duration of the experiment, which possibly did not allow for the growth of grazer populations. Moreover, the low efficiency of grazing may also have influenced the results, and it could be related to the selective pressure on better competitors and/or the morphology of prey, which did not favor grazing (Straile, 1997). For instance, phytoplankton may present protuberances, processes and spines (Padisák *et al.*, 2003) and, in some extreme cases such as cyanobacteria, they present mucilage, which gives them the ability to survive through the digestive system of grazers (Porter, 1976).

The positive and significant relationships between the abundance of zooplankton, rotifers, cladocerans and copepods and the biovolume of MBFG VII, including cyanobacteria (Kruk *et al.*, 2010; Dias & Huszar, 2011), may be related to the presence of mucilage and/or the size of the colony, which are effective strategies to reduce biomass loss by grazing (Kruk *et al.*, 2017). The reduced grazing of these organisms is a result of the decreased efficiency of the filtering apparatus of grazers (Bonecker *et al.*, 2007), which explains the remarkable increase of abundance and biomass for this group of algae in all treatments, when compared to the lake.

The increase in abundances of cladocerans and copepods was related to the increase in the biovolume and abundance of MBFG V, represented by unicellular flagellates of medium to large size (up to 11.6 µm). Among microcrustaceans, copepods are selective regarding the type of food and are capable of manipulating phytoplankton particles, selecting high-quality cells and eliminating toxic components or those algae that do not provide a high energy gain (DeMott *et al.*, 1991; Russo *et al.*, 2016). An experimental study showed a strong and significant relationship between autotrophic nanoflagellate and copepod abundances, which was attributed to the selective grazing by copepods on medium-sized (20-40 µm) ciliates, representing a potential predator for these flagellates (Zöllner, *et al.*, 2003). The biomass of these microcrustaceans also increased where the biovolume and abundance of MBFG V were important to the phytoplankton community, and the zooplankton:phytoplankton ratio was higher.

MBFG IV, represented by medium-sized algae that are palatable for zooplankton, also showed an increased biovolume in most of the treatments. These algae present high energy gain and are potentially predated by cladocerans (Rubenstein & Koehl, 1977; Colina *et al.*, 2016), which also increased in abundance, when compared to the lake, mainly in the higher grazing treatments. The increase in cladoceran abundance probably influenced the decreased abundance of rotifers and copepods in the same treatments.

Positive and significant relationships observed among cladoceran and copepod abundance and MBFG I biovolume was observed due to the predator-prey relationship. These algae are considered as microphages (very small - up to 5 µm - with a high surface/volume ratio), and shwould probably be a preferential prey for cladocerans, represented mainly by small body-size species, and copepods, represented mainly by nauplii. The size of the particles consumed by zooplankton is directly related to their body size (Özkan *et al.*, 2014; Bomfim *et al.*, 2018).

CONCLUSION

Rotifers dominated the zooplankton community, but their abundance decreased at the end of the experiment, as did the abundance of copepods, while cladoceran abundance increased. The findings suggested competition among these species. Copepods were represented mainly by nauplii and cladocerans by small body-size species. The zooplankton composition did not change in the experiment. The increase in the abundance and biovolume of phytoplankton was related to bottom-up mechanisms, and the change in the MBFGs' contribution was related to the increase in the abundance of zooplankton and groups, competition among grazers and exploitative competition among MBFGs. Only rotifers showed a grazer gradient pressure on small cyanobacteria. Other MBFGs provided a food resource for the microcrustaceans, but their abundance did not decrease. The results allowed us to corroborate our hypothesis partially, and the experiment contributes to the knowledge on an important part of the trophic dynamics among planktonic communities in subtropical floodplain shallow lakes.

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Zooplankton advective losses may affect chlorophyll-*a* concentrations in fishless high-mountain lakes

Rafael Morales-Baquero[1,*], Carmen Pérez-Martínez[1], Eloísa Ramos-Rodríguez[1], Pedro Sánchez-Castillo[2], Manuel Villar-Argaiz[1] and José María Conde-Porcuna[1]

[1] Departament of Ecology and Institute of Water Research, University of Granada. 18071 Granada, Spain.
[2] Departament of Botany, University of Granada. 18071 Granada, Spain.

[*] Corresponding author: rmorales@ugr.es

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ABSTRACT

Zooplankton advective losses may affect chlorophyll-*a* concentrations in fishless high-mountain lakes

Hydraulic washout of lakes and reservoirs is recognized as a major regulating factor of both, phyto- and zooplankton populations. We have analysed the changes in the chlorophyll-*a* concentration in 21 high-mountain lakes from Sierra Nevada. Eleven lakes proved to have superficial diffuse inlets and outlets (open lakes) while the other 10 have no outlets, or no permanent outlets (closed lakes), where the plankton losses by washout are not possible. The lakes were sampled on two occasions during the ice-free season: just after the spring thaw (July) and by late August, after plankton development. In July, chlorophyll-*a* was quite similar between the closed and open lakes while in August the open lakes had about three-fold more chlorophyll-*a* than did closed ones. Model selection analysis made with 12 variables than can affect chlorophyll-*a* indicated that, in July, chlorophyll-*a* was related mainly to the maximum depth of closed lakes although the type of lake (closed or open) had no effect. However, in August, the type of lake (more chlorophyll-*a* in open than in closed lakes) and the ratio between dissolved inorganic nitrogen and total phosphorus (DIN:TP ratio) (higher values, lower chlorophyll-*a*), was related to chlorophyll-*a* concentration. The results of DIN, soluble reactive phosphorus (SRP) and nutrient ratios did not indicate a different nutrient availability between open and closed lakes in either of the two periods considered. Nevertheless, the data available showed that the zooplankton was, on average, about two-fold more abundant in the closed lakes than in the open ones during the entire ice-free season. These results indicate that the advective losses caused by the water flow could be greater for the zooplankton than for the phytoplankton, interfering with the coupling of trophic chains. Thus, part of primary production in the open lakes could not be transferred to higher trophic levels because of the zooplankton losses.

Key words: High-mountain lakes, advective losses, zooplankton, phytoplankton, chlorophyll-*a*, grazing pressure, phytoplankton control

RESUMEN

*Las pérdidas advectivas de zooplankton pueden afectar al contenido de clorofila-*a* en lagos de alta montaña sin peces*

*El desagüe de lagos y embalses es un factor importante que se reconoce como regulador de las poblaciones de fito y zooplancton. En este trabajo analizamos los cambios en las concentraciones de clorofila-*a *en 21 lagunas de alta montaña de Sierra Nevada, once de las cuales presentan salidas superficiales de agua permanentes (lagunas abiertos) mientras las otras diez no presentan salidas superficiales, o sólo en un corto periodo (lagunas cerradas), en las que la pérdidade plancton por desagüe no es posible. Las lagunas se muestrearon en dos ocasiones durante la estación libre de hielo: en julio, justo tras el deshielo primaveral y al final de agosto, cuando el plancton se ha desarrollado. En julio, la concentración de clorofila-*a *fue similar en lagunas abiertas y cerradas, mientras que en agosto las lagunas abiertas presentaron aproximadamente tres veces más clorofila-*a *que las cerradas. Un análisis de selección de modelos efectuado sobre 12 variables que pueden afectar a la clorofila-*a *reveló que en julio la clorofila-*a *se relacionó con la profundidad de las lagunas cerradas, pero el tipo de laguna (abierta o cerrada) no tuvo ningún efecto sobre aquella. Sin embargo, en agosto, el tipo de laguna (más clorofila-*a *en las lagunas* *abiertas que en las cerradas) y la razón nitrógeno inorgánico disuelto:fósforo total (DIN/TP) (a valores más altos, menos clorofila-*a*), mostraron efecto sobre los niveles de clorofila-*a*. Los valores del DIN, del fósforo reactivo soluble (SRP) y razones de nutrientes no indican diferencia en la disponibilidad de nutrientes entre lagunas abiertas o cerradas en ninguno de los períodos considerados. No obstante, los datos disponibles muestran que la abundancia de zooplancton es, en promedio, el doble en las lagunas cerradas que en las abiertas durante un periodo de deshielo completo. Estos resultados señalan que las pérdidas advectivas producidas por el flujo de agua pueden ser más importantes para el zooplancton que para el fitoplancton, interfiriendo en el acoplamiento de las cadenas tróficas. Así, parte de la producción primaria de las lagunas no puede transferirse a niveles tróficos superiores debido a las pérdidas de zooplancton.*

Palabras clave: *Lagos alta montaña, pérdidas advectivas, clorofila-*a*, fitoplancton, zooplancton, presión de herbivoría, control del fitoplancton*

INTRODUCTION

Phytoplankton abundance is presumed to represent a balance between *in situ* growth (affected mainly by temperature, light, and nutrient availability) and loss processes, which may include sinking, grazing, parasitism, or washout. Zooplankton abundance is also a balance between growth (affected primarily by temperature and algal availability) and loss processes such as predation, parasitism, and washout. Thus, hydraulic washout is recognized as an important regulating factor of both, phyto- and zooplankton populations (Reynolds, 1984; Wetzel, 2001; Kalff, 2002) and the models for estimating plankton population growth include this parameter modulating the plankton growth rate (Reynolds, 1984; Lucas *et al.*, 2009). Hence, in the absence of others factors, plankton population abundance increases when population growth rate is faster than hydraulic loss and decreases when washout is faster than growth rate. Therefore, in a specific system, the effect of advective losses through lake out-flow is presumably greater on zooplankton, regardless of its size and swimming capacity, than phytoplankton, as the generation time of the former is significantly longer (Wetzel, 2001).

In this regard, some studies suggest that slower reproducing zooplankton may be more susceptible than phytoplankton to advective losses. For example, advective losses appear to be important in explaining differences between planktonic communities in lentic and lotic environments in the studies of Pace *et al.* (1992) and Thorp *et al.* (1994). Moreover, there is evidence that zooplankton abundance is positively correlated to water residence time but not with the phytoplankton abundance in rivers (Basu & Pick, 1996), reservoirs (Campbell *et al.*, 1998; Beaver *et al.*, 2013) or rapidly flushing lakes (Walz & Welker, 1998). Also, according to Havel *et al.* (2009) the observed decline in crustacean zooplankton downstream from dams reflects that reproduction is insufficient to balance high mortality by advective losses in the channel, where high algal biomass and rapid population growth rates allowed rotifers to dominate there.

For lakes with relatively similar morphometry, water residence time should be contrastingly lower in lakes with outlets (open lakes) than lakes without outflows or with no permanent ones (closed lakes). Consequently, advective losses should be greater in open lakes for zooplankton than for phytoplankton. For that reason, with similar resource availability for phytoplankton and without zooplankton predators, phytoplankton biomass should be less in closed lakes than in open lakes. Hence, we hypothesised that grazing pressure on phytoplankton should be higher in closed lakes with respect to open ones if no mechanism other than advection is removing zooplankton from the lakes. In this work, we compare the phytoplankton development in a group of 21 small, fishless, high-mountain lakes in Sierra Nevada (Spain) to test the aforementioned hypothesis.

METHODS

Site description

In the Sierra Nevada mountains of SE Spain ((36º 55' - 37º 15' N, 2º 31' - 3º 40' W; maximum altitude 3482 m a.s.l), ~50 small lakes of glacial

Table 1. Maximum depth, chlorophyll-*a* values and DIN:TP ratios in the Sierra Nevada lakes studied in July and August. Zooplankton, Rotifera, and Crustacea abundances averaged for an entire ice-free season are from Morales-Baquero & Conde-Porcuna (2000) (n: times sampled each lake). *Profundidad máxima, concentración de clorofila-*a *y cociente DIN:TP en los lagos estudiados de Sierra Nevada en julio y agosto. Los valores de abundancia media de zooplancton para el período libre de hielo están tomados de Morales-Baquero & Conde-Porcuna (2000) (n: número de veces que se muestreó cada lago).*

Lake	Location UTM (30S)	Outflow[1]	Lake surface area (ha)	Maximun depth (m)	Chl-*a* (µg/L)		DIN:TP (weight)		Zooplankton[2] (ind/L)	Rotifera[2] (ind/L)	Crustacea[2] (ind/L)	n
					July	August	July	August				
Virgen 2	VG664009	1	0.01	1.3	0.34	4.10	6.6	1.2	12.24 (7.13)	0.55 (0.16)	11.69 (6.57)	5
Yeguas	VG662013	1	0.33	2.5	1.22	3.21	49.5	2.6	-	-	-	
Lanjarón 2	VF641991	1	0.05	1	0.30	1.93	3.1	1.0	93.40 (53.84)	28.94 (26.76)	64.47 (48.91)	3
Mosca	VG723017	1	0.44	2.8	0.60	3.68	31.0	6.3	0.83 (0.32)	0.13 (0.08)	0.70 (0.28)	3
Gemela	VG714004	1	0.07	0.3	0.43	2.31	19.1	0.9	20.02 (9.06)	1.58 (0.72)	18.44 (10.23)	6
Aguas Verdes	VG674006	1	0.19	2.8	0.34	0.83	4.6	2.7	10.59 (5.64)	0.60 (0.34)	9.99 (5.97)	4
Siete lag. 2	VG735014	1	0.34	3.5	0.51	0.51	13.8	2.9	58.30 (35.69)	2.44 (1.34)	55.86 (35.83)	6
Siete lag. 4	VG737012	1	0.19	0.5	0.86	0.58	21.8	6.1	2.14 (0.42)	0.44 (0.19)	1.70 (0.44)	6
Borreguil	VG734009	1	0.18	2	1.11	2.31	14.1	2.7	0.98 (0.16)	0.74 (0.20)	0.24 (0.11)	4
Siete lag. 7	VG739004	1	0.53	0.8	0.38	1.15	14.8	4.4	5.10 (1.85)	0.32 (0.07)	4.78 (3.34)	6
Peñón Negro	VF738983	1	0.67	2	2.82	5.39	6.20	0.6	47.22 (17.71)	21.70 (18.75)	25.52 (11.36	4
Lanjarón 1	VF646993	1-0	0.29	2.5	-	1.09	-	0.8	111.98 (73.48)	109.08 (108.73)	2.90 (1.31)	3
Majano	VG712003	1-0	0.27	0.8	0.68	3.21	24.1	1.1	54.26 (31.49)	36.24 (32.55)	18.02 (8.01)	5
Rio Seco	VG694009	1-0	0.42	2	0.47	0.46	6.0	1.9	89.06 (52.30)	1.48 (0.92)	87.59 (51.73)	4
Rio Seco Inf.	VG697008	1-0	0.12	0.5	3.21	2.30	3.3	0.5	101.17 (43.25)	94.35 (42.44)	6.82 (4.61)	3
Larga	VG704017	0	1.77	7	0.21	0.43	26.6	-	22.88 (14.23)	0.98 (0.78)	21.90 (13.55	2
Rio Seco Sup.	VG692008	0	0.07	1.5	0.97	0.83	11.2	1.9	18.97 (6.85)	7.24 (6.84)	11.73 (5.25)	4
Lanjarón 3	VF636985	0	0.11	1.5	1.07	2.70	1.0	0.6	78.65 (25.40)	73.65 (29.35)	5.00 (4.15)	2
Caldera	VG708012	0	2.10	7	0.21	0.25	54.4	5.7	15.87 (5.05)	0.88 (0.41)	14.99 (5.48)	5
Caballo	VF612968	0	0.48	4	0.17	0.38	1.3	1.5	54.08 (30.675)	9.30 (8.90)	44.78 (39.35)	2
Cuadrada	VF618975	0	0.24	5	0.21	0.77	3.3	2.2	54.875 (42.6)	28.30 (2.80)	26.58 (14.58)	2

1) 1: Open lakes; 0: Closed lakes; 1-0: lakes open in July and closed in August

2) Averages (standard error); n= number of samples

origin lie at an elevation of ~2800–3100 m a.s.l. These mountain lakes are typically small (surface area < 1 ha) and shallow (maximum depth < 10 m) and may undergo variable reductions in their water level depending on annual meteorological conditions. In this study, the lake surface area and maximum depth ranged from 0.01 to 1.0 ha and 0.3 to 8 m, respectively (Table 1). Further details can be found in Morales-Baquero *et al.* (1999). The lakes are fishless and Secchi disk visibility exceeds the water depth. Normally these water bodies remain ice-covered from November to June and do not thermally stratify during the summer. Lake waters are relatively soft: conductivity ranged from 5 to 77 µS/cm; total alkalinity from 50 to 400 µeq/L and pH from 6.5 to 9.5 (mean values at the time of this study). The phytoplankton is dominated by nannoplanktonic species (Sánchez-Castillo *et al.*, 1989) and the dominant zooplankton species are *Mixodiaptomus laciniatus* and *Diaptomus cyaneus* among copepods, *Daphnia pulicaria* among cladocerans and *Hexarthra bulgarica* and *Euchlanis dilatata* among rotifers (Cruz-Pizarro, 1983, Morales-Baquero *et al.*, 1989).

Sampling and analyses

A total of 21 lakes were studied (Table 1). The lakes were sampled just after the ice thaw and later, in the middle of the growing season, when the plankton communities were well developed. On each occasion, we collected the samples from all the lakes in the shortest time period possible. The first sampling took place over a 12-day period between 15-27 July 1991, and the second over a 13-day period between 21 August and 3 September of the same year. Hereafter, these periods will be referred to as "July" and "August", respectively. Samples were taken at the deepest point of each lake. An equal volume of water was extracted (using a centrifugal electric pump) from four evenly spaced levels of the water column, pre-filtered through a 40-µm NYTAL® net to remove the zooplankton, and then mixed together. Aliquots for analysis were taken from this mixed sample, and those for chemical analysis were stored in the dark at 4 ºC and analysed within 24 h of sampling.

Chlorophyll-*a* was determined by filtering 1 L of water through a GF/C filter, immediately after collection. The filter was placed in a glass vial, 10 ml of 95 % methanol was added, and the vial was stored in the dark at 4 ºC during transport. The vial was then frozen at -10 ºC for 24 h and the extract measured and corrected for pheopigments using a Hewlett Packard scanning spectrophotometer. Total nitrogen (TN) and total phosphorus (TP) were determined in unfiltered aliquots of the water samples, digested using a mixture of potassium persulphate and boric acid at 120 ºC for 30 min, and subsequently analysed by the ultraviolet method (APHA, 1989) and the ascorbic-acid method of Murphy & Riley (1962), respectively. SRP (soluble reactive phosphorus) and DIN (NH_4^+-N + NO_2^--N + NO_3^--N) were analysed from 0.45-µm (GF/C filters) filtered aliquots and determined using the Murphy & Riley (1962), the blue indophenol method (Rodier, 1990), the Strickland & Parsons (1968) and the sodium salicylate method (Rodier, 1990), respectively. According to Morris & Lewis (1988), we used several indexes (DIN:TP, DIN: SRP and TN:TP) to examine the nutrient limiting for phytoplankton growth. Concentrations and elemental ratios are reported by weight.

The zooplankton of the lakes included in the present study was previously quantified in a more detailed sampling program (Morales-Baquero, 1985). The lakes were sampled in 1981 and 1982, during the entire ice-free period but more intensively in 1982 (four times in each lake in most cases; see Table 1). A total of 20 L of water from the littoral zone were filtered through 40-µm mesh. Zooplankton average abundance of rotifers and crustacean [cladocerans + copepods (nauplii, copepodites and adults)] are shown in Table 1, and were previously published in Morales-Baquero & Conde-Porcuna (2000).

Statistical analyses

Statistical analyses were performed using program R 3.4.0 (R Foundation for Statistical Computing). We analysed the relationship of chlorophyll-*a* with the environmental variables that might affect it. The 12 environmental variables include lake morphometric data (type of basin [open or closed], maximum depth, lake surface, catchment area, and the relation of lake surface to the catchment area), nutrient availability [dissolved inorganic nitrogen (DIN), soluble reactive phosphate (SRP), total nitrogen (TN) and total phosphorus (TP)], nutrient ratios (DIN:SRP, DIN:TP and TN:TP) and other environmental variables such as temperature and presence of littoral vegetation. The categorical variable "type of basin" indicated the presence of outlets in both sampling periods (open lakes) or no permanent or absence of outlets (closed lakes). In the last block, we included four lakes that had weak outlets in July and were closed in August. These lakes showed no significantly different pattern from those of the rest of closed lakes. In both open and closed lakes the inflows were diffuse. Continuous environmental variables were log-transformed (base 10) prior to statistical analyses in order to achieve normality and homoscedasticity as well as to linearize their relationships.

The environmental variables related to the chlorophyll-*a* concentration were assigned by model selection. We also included in the models the interactions of the type of basin with the other environmental variables. The R package "MuMIn" (Bartoń, 2018) was used to rank models, and two model-selection tests were performed, one with July data and the other with August data. Previously, high collinear variables (Variance Inflation Factor (VIF) > 10) were excluded from the set of variables of each model using the R package "usdm" (Naimi *et al.*, 2014). Model selection was based on the second-order Akaike information criterion (AIC_c) because of the relatively small sample sizes (Burnham & Anderson, 2002). If differences in AIC_c between each model and the model with the minimum AIC_c (ΔAIC_c) is lower than 2, the models have relatively equal support (Burnham & Anderson, 2002). Additionally, the Akaike weights were summed (cumulative AIC_c weights) over all possible models containing a given variable to measure the relative importance of each independent variable (Burnham & Anderson, 2002; Burnham & Anderson, 2004). Variables with a cumulative weight ≥ 0.5 show strong evidence of inducing a response in the dependent variable according to Barbieri and Berger (2004). Assumptions of linear models (normality of residuals, homoscedasticity, outliers and no multicollinearity) were checked for the best models.

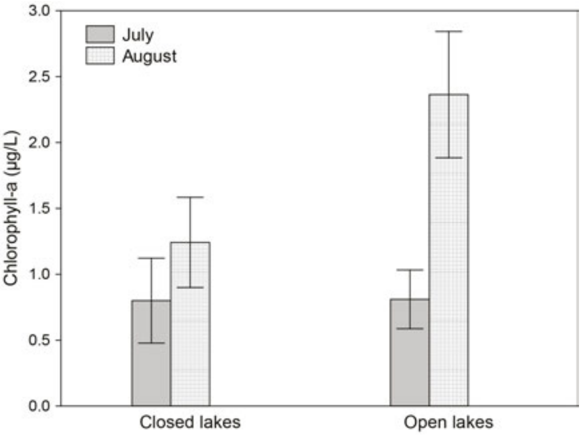

Figure 1. Mean value of chlorophyll-*a* concentrations in the open and closed lakes in July and August of 1991. Whiskers show the standard error. *Valor medio de la concentración de clorofila-*a *en las lagunas abiertas y cerradas en julio y agosto de 1991. Las líneas de dispersión muestran el error estándar.*

We also used an unpaired t-test to analyse differences in the zooplankton abundance averaged for the ice-free period between open and closed lakes. Because zooplankton abundance data showed a mean-variance relationship according to Taylor power law, these data were powered to ¼ (Downing, 1979).

RESULTS

In July the chlorophyll-*a* concentrations were quite similar between open and closed lakes while in August open lakes showed some three-fold more chlorophyll-*a* than did closed ones (Fig. 1). Model selection with July data showed that variables with the highest cumulative AIC_c weights were the type of basin (0.84), depth (0.72), and the interaction between the type of basin with depth (0.66). Consequently, these variables had the highest relative importance. Other variables with AIC_c weights higher than 0.5 were the ratio TN:TP (0.62) and the interaction between type of basin and the ratio TN:TP (0.53). In fact, the best selected model includes those five variables (ΔAIC_c > 4 with

Table 2. Best model for the effect of environmental variables on the chlorophyll-*a* concentration of lakes in July and August. Statistically significant values ($p < 0.05$) are indicated in bold. SE = Standard error. *Mejor modelo obtenido para el efecto de las variables ambientales sobre la concentración de clorofila-*a *en las lagunas en julio y en agosto. Los valores estadísticamente significativos (*p *< 0.05) se indican en negrita. SE = error estándar.*

July	$AIC_c = 15.24$			
	Estimate	SE	t value	*p*
Intercept	0.042	0.126	0.338	0.740
Type of basin (open)	-0.279	0.154	-1.806	0.092
Depth$^+$	-1.009	0.289	-3.495	**0.004**
Type of basin x Depth	1.255	0.381	3.294	**0.005**
August	$AIC_c = 14.56$			
	Estimate	SE	t value	*p*
Intercept	0.078	0.101	0.778	0.447
Type of basin (open)	0.438	0.137	3.199	**0.005**
DIN:TP ratio$^+$	-0.726	0.205	-3.544	**0.002**

$^+$log transformed

respect to the rest of the models). However, that model has too many variables for the number of cases we had, and some VIF values in the model were higher than 10. The second-best model includes only the three variables with the highest AIC_C weights and, in this case, VIF values were low (< 3) (Table 2). According to that model, the maximum depth of closed lakes could exert a negative effect on chlorophyll-*a* (Table 2).

Model selection with August data showed that the only variables with cumulative AIC_C weights higher than 0.5 were the ratio DIN:TP (0.96) and the type of basin (0.82), which suggest that these variables were the most relevant variables to explain the chlorophyll-*a* concentrations. Although 9 models were selected with a $\Delta AIC_C < 2$, the best model with significant slopes and assumptions was the model including only the DIN:TP and the type of basin (Table 3, Fig. 2). The slopes of the regression lines linking the DIN:TP ratio and chlorophyll-*a* in both types of lakes did not significantly differ ($p > 0.05$), but the highly significant differences in the interception points of the two lines suggest a clear effect of the outflow in the chlorophyll-*a* content. According to this model, open lakes had a higher chlorophyll-*a* concentration than did closed lakes for similar DIN:TP ratios (Table 2, Fig. 2).

For contrasting purposes, when we used those variables (type of basin and the ratio DIN:TP) to model chlorophyll-*a* concentration with July data, we found no significant relationships. Consequently, in August, when the algal and zooplankton communities have had enough time to develop, the phytoplankton shows a higher biomass in open lakes than in closed ones regardless of the nutrient supply. The latter results agree with the idea of lower grazing pressure in the open lakes due to washout losses of zooplankton. This idea is also supported by the available data on the zooplankton of Sierra Nevada lakes (Table 1). Figure 3 shows the abundance of zooplankton in open and closed lakes averaged for the ice-free period. Rotifers has an average of 36.2 ind/L in closed lakes

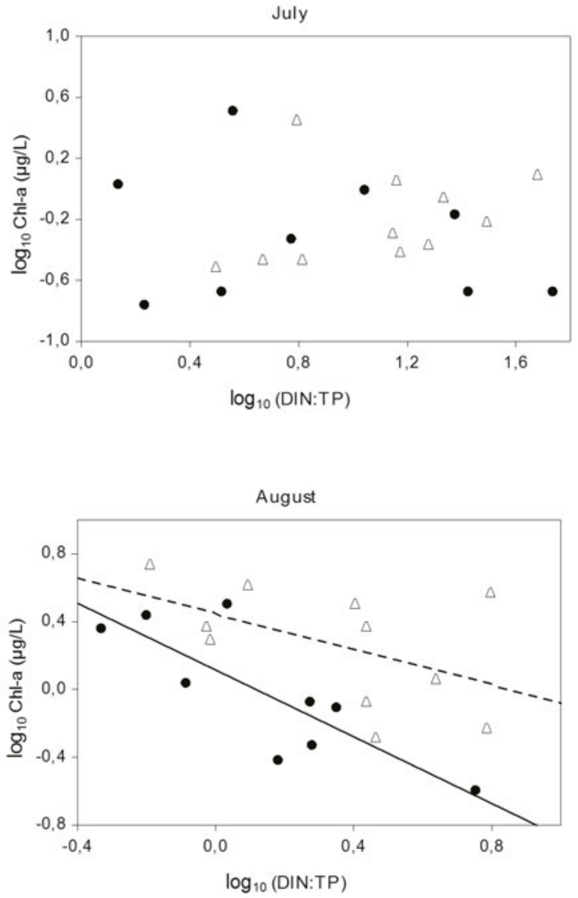

Figure 2. Relationships between the DIN:TP ratio and the chlorophyll-*a* concentrations in open and closed lakes in a) July and b) August. Significant regression lines are shown for open (triangles) and closed (solid circles) lakes in August. *Relación entre el cociente DIN:TP y la concentración de clorofila-*a *en las lagunas abiertas y cerradas en a) julio y b) agosto. Se muestran las líneas de regresión significativas para lagunas abiertas (triángulos) y cerradas (círculos) en agosto.*

which is about 6-fold more than in open lakes (5.7 ind/L) and this difference is statistically significant (unpaired t-test: t = 2.62; $p = 0.019$). Also, the average crustacean abundance tended to be higher in closed lakes (24.0 ind/L) than in open ones (19.3 ind/L), although not statistically significant. Altogether, the closed lakes (55.5 ind/L) had more than twice the average zooplankton abundance of the open lakes (24.1 ind/L), this difference being statistically significant (unpaired t-test: t = -2.85; $p = 0.011$; n = 10 lakes in each group).

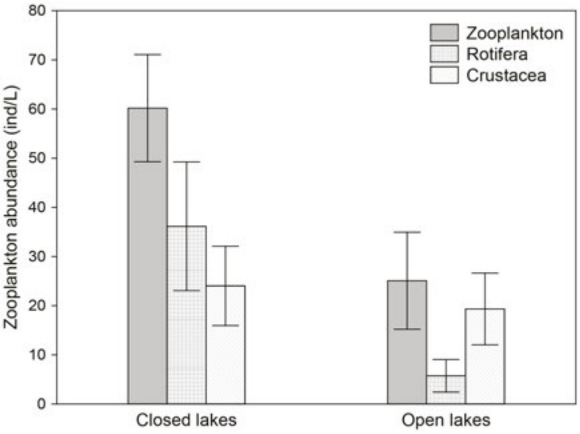

Figure 3. Averaged zooplankton, Rotifera and Crustacea abundance for the entire ice-free period (see Table 1) in open and closed lakes. *Abundancia media de zooplancton, rotíferos y crustáceos para el período libre de hielo (ver Tabla 1) en lagunas abiertas y cerradas.*

DISCUSSION

The results of model selection from the 12 variables considered that can affect phytoplankton provided evidence that both the nutrient availability and the type of basin (open or closed) simultaneously controlled the phytoplankton biomass in the lakes of Sierra Nevada in August. The effect of the factor “type of basin” can be related to the grazing pressure of the zooplankton, which can be altered by the advective transport.

In July, when the planktonic community had not yet developed, there was a negative relationship of depth with chlorophyll-*a* in closed lakes, i.e. greater phytoplankton densities in shallow closed lakes in comparison to deeper closed ones. It is possible that in the shallow lakes, having less water volume, less time is needed to recolonize the pelagic zone from the sediment by the overwintering resting forms. The lack of a relationship between the chlorophyll-*a* and DIN:TP ratio in both types of lakes in July, when chlorophyll-*a* levels were low, was likely the result of a low nutrient limitation at this time. Seasonal changes in the strength of nutrient limitation in lakes have been reported elsewhere (Elser *et al.*, 1995). Particularly, nutrient limitation is often more relevant during the summer in non-stratified lakes

(Hansen *et al.*, 1997). Seasonal studies in the Sierra Nevada lakes have also shown evidence of changes in nutrient limitation from the beginning of the ice-free period to the middle of the growing season when the plankton has developed (Morales-Baquero *et al.*, 1999). In fact, bioassays have demonstrated that nutrient limitation is greater during the middle than at the beginning of the growing season (Villar-Argaiz, 1999).

In August, once the plankton has been developed, nutrient limitation could be stronger in all lakes and the relationship between the relative N and P availability and the phytoplankton abundance could be apparent in these lakes. N limitation appears when DIN values are very low (< 6 µg-N/L; Morris & Lewis, 1988) and DIN:TP values are below 3.4 (by weight) (Bergström, 2010). In August the DIN:TP ratios decreased in all lakes (Table 1) and the continuous negative relationship between chlorophyll-*a* and DIN:TP ratios in this month but not in July (Fig. 2) could reflect an increment of the degree of nutrient limitations as summer progresses and phytoplankton populations grow. Despite that this limitation appeared to occur similarly in both open and closed lakes, according the model performed, open lakes had a greater chlorophyll-*a* concentration than did closed lakes for similar DIN:TP ratios. Thus, the observed higher chlorophyll-*a* contents in the open lakes in comparison to the closed ones does not appear to depend on differences in nutrient supply.

Another factor that can cause differences in phytoplankton abundances is sedimentation below the euphotic zone, as light tends to be limited with depth. Since the systems with high water residence time can increase the rates of sedimentation of algae and light limitation (Søballe & Bachmann, 1984), phytoplankton losses due to sedimentation should be higher in the closed lakes than in the open ones. Nevertheless, this does not plausibly explain the lower chlorophyll-*a* contents observed in the closed lakes with respect to open ones, because in Sierra Nevada the light generally reaches the lake bottom.

Higher grazing pressure of zooplankton on phytoplankton in closed lakes is a more plausible explanation for the observed trends. Evidence indicates that changes in the outflow losses of zooplankton can change the standing stock of phytoplankton without alterations in the trophic status of the systems. For instance, Campbell *et al.* (1998) showed that after the conversion of a rapidly flushing lake in a reservoir, with much longer water residence time, the zooplankton biomass increased by one order of magnitude while the phytoplankton biomass fell below former values. These changes occurred independently of primary production or nutrient availability. Moreover, Morales-Baquero *et al.* (1994) has shown a negative relationship between water residence time and the chlorophyll-*a* per zooplankton biomass in reservoirs of contrasting trophic conditions. This is further evidence of high zooplankton grazing pressure over phytoplankton in waters with longer residence time.

The positive relationship between the water residence time and zooplankton abundance is well reported in rivers (Basu & Pick, 1996), lakes (Walz& Welker, 1998; Rellstab *et al.*, 2007), floodplains (Bozelli, 1994; Dias *et al.*, 2017), and reservoirs (Campbell *et al.*, 1998; Beaver *et al.*, 2013), pointing out the importance of the advective losses of zooplankton. In fact, the export of zooplankton through the outflow of reservoirs can be a major source of food for the fish community of the waters (Akopian *et al.*, 1999). Also, the increase in lake water residence time has been indicated as a possible explanation of the observed long-term trends of *Daphnia* increases associated with warming in Sierra Nevada lakes (Jiménez *et al.*, 2015, 2018)

In Sierra Nevada, zooplankton advective losses can occur in open lakes but not in closed ones, and the available data supports this contention. The zooplankton data in Table 1 are from a robust sampling survey, and the composition and relative abundance of the zooplankton communities in the lakes is quite similar throughout several study years (Carrillo *et al.*, 1996, Morales-Baquero *et al.*, 2006, Pérez-Martínez *et al.*, 2007). Hence, although zooplankton and phytoplankton were not sampled in the same year, we can attribute higher zooplankton predation pressure on phytoplankton in closed lakes than in open ones. Furthermore, the zooplankton samples were taken from the littoral zone. In this zone, the effect of advection over the zooplankton is probably less influential than in the open waters. In fact, contrary to high flushing rate habitats, inshore habitats act as storage zones for zooplankton (Reckendorfer *et al.*, 1999; Walks, 2007); nevertheless, the open lakes in Sierra Nevada contain less zooplankton than do closed ones. On the other hand, we found more zooplankton in closed lakes, despite that littoral sampling probably underestimates some populations, such as *Daphnia* sp., which tend to avoid the shore (Ringelberg, 1969) and have a strong grazing impact on phytoplankton. Thus, as expected from our initial hypothesis, the lower phytoplankton biomasses in the closed lakes in comparison to the open ones, for similar nutrient availability, can be attributed to a high grazing pressure due to lack of advective losses of zooplankton in the closed lakes.

In summary, this study indicates that in the small lakes of Sierra Nevada, outflow can interfere in the phytoplankton-zooplankton coupling. Our results suggest that, in the open lakes, part of the primary production cannot be transferred to higher trophic levels because of zooplankton losses. Advective losses are great in small lakes, but also in reservoirs with low water residence times because of high rates of water extraction. This abiotic mechanism of rarefaction can interfere with the articulation of the trophic chains and, together with other factors, can help to explain failures of the trophic-cascade hypothesis (Carpenter *et al.*, 1985) when it is applied to reservoirs in comparison to natural lakes.

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Limited importance of primary production in the deep chlorophyll layer for macro-zooplankton in an oligotrophic karst lake: A whole-lake ^{15}N experiment

Wayne A. Wurtsbaugh[1,*], María Rosa Miracle[2,†], Antonio Camacho[2], Javier Armengol[2] and Eduardo Vicente[2]

[1] Watershed Sciences Department/Ecology Center, Utah State University, Logan, UT 84322-5210, U.S.A.
[2] Cavanilles Institute for Biodiversity and Evolutionary Biology, University of Valencia, E-46980 Paterna, Spain.

* Corresponding author: wayne.wurtsbaugh@usu.edu

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ABSTRACT

Limited importance of primary production in the deep chlorophyll layer for macro-zooplankton in an oligotrophic karst lake: A whole lake ^{15}N experiment

Deep-chlorophyll layers (DCL) in oligotrophic lakes contribute significantly to primary productivity, but the importance of this production for the rest of the food web and for other strata is unknown. In Laguna El Tejo, a sheltered 1.7-ha Spanish karst lake, chlorophyll levels were < 2 µg/L in the epilimnion but reached 10 µg/L in the metalimnion and upper hypolimnion where cyanobacterial picoplankton dominated. Particulate nitrogen levels were 2-14 times higher in the metalimnion than in the epilimnion and 42 % of the primary productivity occurred in the deeper strata where the DCL was located. To address the trophic importance of the high biomass and production in the deep chlorophyll layer, we injected $^{15}NH_4^+$ and rhodamine into a 0.5-m strata (15-16 m) in the metalimnion of the lake. The $^{15}NH_4^+$ taken up by the nitrogen-limited phytoplankton allowed us to measure the importance of biologically mediated transport whereas the rhodamine traced physical eddy diffusion. After 28 days 55 % of the ^{15}N could be accounted for: 71 % remained in the metalimnetic seston (11-18 m), 10 % was in the hypolimnetic seston (18-20 m), 11 % was found above in the epilimnetic seston, and only 8 % had sedimented into the anoxic layer below 20 m. Only negligible amounts of rhodamine (corrected for degradation) moved beyond the 14-18 m strata in the lake, but bio-diffusivity of ^{15}N was 3 times greater than the physically induced diffusivity of rhodamine. A mixing model indicated that the deep chlorophyll layer contributed only 1-2 % of the diet of epilimnetic macrozooplankton but 14-33 % of the diet of the meta-hypolimnetic zooplankton. The data indicate the overall importance for primary production and the sequestration of nutrients in the DCL, but relatively limited importance for the macrozooplankton in the lake.

Key words: deep chlorophyll maxima, DCM, diffusivity, tracer

RESUMEN

Relevancia limitada de la capa profunda de clorofila para la producción del del macrozooplancton en un lago kárstico oligotrófico: un experimento de adición de ^{15}N en todo el lago

En lagos oligotróficos los máximos profundos de clorofila (Deep-chlorophyll layers, DCL) contribuyen significativamente a la producción primaria, pero se desconoce la importancia relativa que tiene esta producción para el conjunto de la red trófica y para otros estratos lacustres. En la laguna del Tejo, una laguna cárstica española de 1,7 ha de superficie, encajada en una dolina que la protege del viento; los valores de clorofila fueron < 2 µg/L en el epilimnion alcanzando valores de 10 µg/L en el metalimnion y en la parte superior del hipolimnion, donde el picoplancton, formado por picocianobacterias, era dominante. Los valores de nitrógeno particulado fueron entre 2 y 14 veces superiores en el metalimnion que en el epilimnion, dándose el 56 % de la producción primaria en los estratos profundos donde se localizaba el DCL. Para investigar la importancia trófica de la elevada biomasa y producción primaria de este máximo profundo de clorofila, inyectamos $^{15}NH_4^+$ y rodamina en un estrato de 0.5-m en el metalimnion (entre 15-16 m). El $^{15}NH_4^+$ tomado por el fitoplancton (que estaba *limitado por N), nos permitió medir la relevancia del transporte biológico; mientras que la rodamina nos permitió trazar la difusión pasiva por causas físicas. Tras 28 días, el destino del 55 % del ^{15}N fue: el 71 % permaneció en el seston metalimnético (11-18 m), el 10 % fue localizado en el hipolimnético (18-20 m), el 11 % más arriba, en el seston del epilimnion, y un 8 % había sedimentado en la capa anóxica, por debajo de 20 m. Únicamente cantidades insignificantes de rodamina (corregida por su degradación) se desplazaron más allá de los 14-18 m en el lago, pero la biodifusividad del ^{15}N fue 3 veces mayor que la difusividad física de la rodamina. Un modelo mixto indicó que el máximo profundo de clorofila contribuyó tan sólo al 1-2 % de la dieta del zooplancton epilimnético, sin embargo, contribuyó al 14-33 % de la del zooplancton meta-hipolimético. Los datos indican la importancia general que para la producción primaria y la captación de nutrientes tiene el DCL, pero también su relativamente limitada importancia para el macrozooplancton en este lago en su conjunto.*

Palabras clave: *máximos profundos de clorofila, zooplancton, difusividad, trazador*

INTRODUCTION

Oligotrophic lakes and oceans usually have zones enriched in phytoplankton, or deep chlorophyll layers (DCL) below the mixed layer (Fee, 1976; Camacho, 2006; Cullen, 2015; Silsbe & Malkin, 2016; Leach *et al.*, 2018). The peak in chlorophyll is frequently referred to as the deep chlorophyll maximum (DCM). DCLs can account for over 50 % of primary production in oligotrophic systems and contain much of the particulate organic matter (seston) in the water column (Silsbe & Malkin, 2016; Giling *et al.*, 2017; Scofield *et al.*, 2020). The high production in the DCL is a consequence of physiological adaptions of the phytoplankton taxa living there (Camacho & Vicente, 1998; Camacho *et al.*, 2001; Camacho, 2006; Cullen, 2015). Much of the research on DCLs has focused on the mechanisms that drive their formation (e.g. Pilati & Wurtsbaugh, 2003; Cullen, 2015; Scofield *et al.*, 2017; Lofton *et al.*, 2020), transport of nutrients between strata (e.g. Pilati & Wurtsbaugh, 2003; Letelier *et al.*, 2004), and their importance as a food source for grazing zooplankton which often migrate between the surface and deep layers (Williamson *et al.*, 1996; Winder *et al.*, 2003). The relative influence of top-down vs. bottom-up control on algal composition and dominance in the DCL varies across phytoplankton groups (Lofton *et al.*, 2020). Additionally, the importance of DCLs for zooplankton feeding in lakes has been debated, with some studies showing them to be very important (Matthews & Mazumder, 2005; Francis *et al.*, 2011; Twiss *et al.*, 2012) whereas others have found a limited role (Wilkinson *et al.*, 2014). Some of this research has focused on diel vertical migration (DVM) of zooplankton that often inhabit the darker, colder water during the day to avoid fish predation, and move into the mixed layer at night, presumably to feed on higher quality foods (Lampert *et al.*, 2003). The underlying assumptions of these migration studies have, however, been questioned (Wurtsbaugh & Neverman, 1988; Williamson *et al.*, 1996; Armengol *et al.*, 2012).

To address some of these questions, we injected a stable isotope of nitrogen ($^{15}NH_4^+$) along with inert rhodamine into the deep chlorophyll layer of a small doline lake in central Spain. The plankton in the lake, Laguna El Tejo, are nitrogen limited (Camacho *et al.*, 2003a), so the tracer was quickly incorporated into the food web. The ensuing isotopic analyses and routine limnological sampling allowed us to address the following questions: (1) How important is the DCL for production processes? (2) What taxa dominate in the DCL? (3) How important is production in the DCL for grazing zooplankton? (4) How fast is biological movement of the ^{15}N in relationship to physical processes that move the rhodamine? (5) What is the half-life of nitrogen in Laguna El Tejo?

MATERIAL AND METHODS

Study site

The experiment was conducted in Laguna El Tejo, located at 39.987° N, 1.878° W, 20 km east of the city of Cuenca, Spain. The circular lake lies at an elevation of 1012 m in a karst depression with an area of only 1.7 ha (Fig. 1A). Steep walls rise 22-50 m above the entire lake so that it is protected from winds and the surface watershed is very small. At the time of the study, the maximum and mean depths of the lake were 26 and 12 m with a volume of 158 000 m^3. No surface inflows enter the lake, and sublacustrine springs and precipitation to the lake surface provide water and nutrients. A capped spring located on the south wall of the depression approximately 30 m above the lake level was sampled occasionally to provide an idea of the nutrient supply in the groundwater. The lake level fluctuates considerably: a morphometric analysis done in 1987 (personal communication, C. Lentisco) indicated that the lake was 5 m deeper than in 1997 when our experiment was conducted and the lake's depth declined approximately 0.4 m during the 231 days we monitored it. The limnology of lakes in this region has been studied extensively (Miracle & Vicente, 1983; Miracle *et al.*, 1992; Armengol & Miracle, 1999; Miracle *et al.*, 2000; Camacho, 2006).

The carbonate rocks in the watershed provide the lake with a pH varying between 8.5-9.1 in the surface waters, and alkalinities near 5.5 meq/L. Lake whitings from $CaCO_3$ precipitation sometimes occur during summer as in the adjacent and better-studied Laguna La Cruz (Rodrigo *et al.*, 1993; Miracle *et al.*, 2000). Laguna El Tejo is oligo-mesotrophic, with summer epilimnetic chlorophyll *a* concentrations varying from 1.5-2 µg/L and Secchi depths from 6-7 m. Dissolved inorganic nitrogen and phosphorus concentrations are near levels of detection in most cases ($P < 0.9$ µg/L; $N < 0.3$ µg/L). Both epilimnetic and metalimnetic phytoplankton change seasonally between nitrogen and phosphorus limitation (Camacho *et al.*, 2003a). Dissolved organic carbon in the epilimnion of the lake usually ranges from 3-4 mg/L. Submerged macrophytes (*Myriophyllum spicatum* and *Chara aspera*) are intermittently present around the steep littoral zone but are not abundant. Introduced minnows (*Luciobarbus guiraonis* and *Achondrostoma arcasii*) are abundant in the lake. The young of both species and adult *A. arcasii* are omnivorous and feed on zooplankton.

^{15}N Tracer Injection and processing

Field collections

We injected $^{15}NH_4Cl$ and rhodamine WT dye into the lake's metalimnion on 17 September 1997 (Fig.1). Prior to the injection 145 g of 10-atom % $^{15}NH_4Cl$ and 652 g of a rhodamine WT impregnated wax cake were mixed together. To inject the tracers, we pumped water up from a depth of 15.5 m with an electric pump and mixed it with the $^{15}NH_4Cl$ and rhodamine. The mixture was then immediately injected at 4-5 L/min through 9 holes in a vertical steel pipe into a 0.5-m thick strata at a depth of 16 m. The tracers

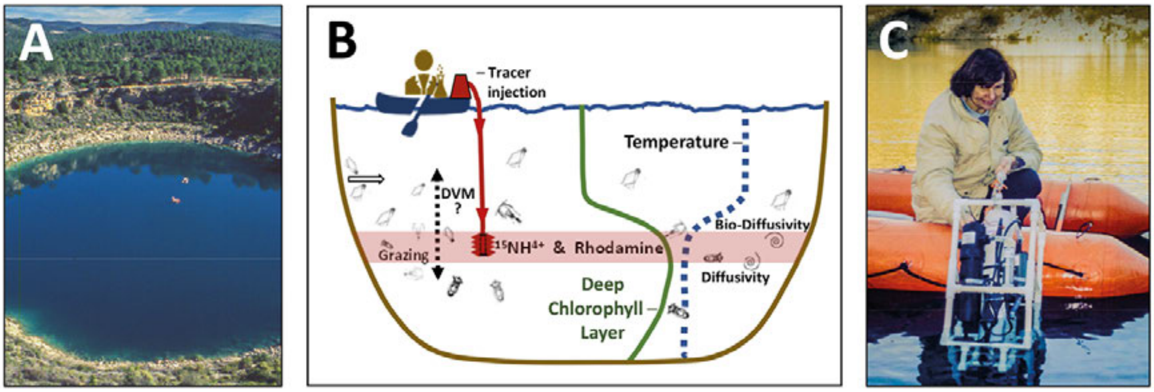

Figure 1. A. Laguna El Tejo with two sampling rafts in upper center. B. Schematic showing tracer injection methods and processes studied. C. Dr. Maria Rosa Miracle sampling with a Seabird salinity-temperature-depth profiler in El Tejo. *A. Laguna del Tejo, con las barcas desde las que se hacia el muestreo. B. Esquema que muestra los métodos de inyección del trazador y el* ^{15}*N, y los procesos estudiados. C. La Profesora María Rosa Miracle, midiendo la salinidad y temperatura con una sonda multiparamétrica en la Laguna del Tejo.*

were injected for ca. 30 min at each of 9 stations located roughly equal-distant around the lake over depths > 20 m. Some limited tracer contamination of the epilimnion likely occurred when the hose and pipe were pulled to the surface without first flushing them with epilimnetic water.

The main experiment was run for 28 days. Samples were obtained from the injection date, September 17th, to October 15th, though measurements of certain variables where taken up to one year later. Seston samples were collected at 7-9 depths between 12:00-19:00 h with a 2.7 L, 50-cm long Ruttner bottle at four stations placed equidistant around the lake over maximum water depths of ca. 23 m. Lake water samples were stored at 4 °C and processed the next day. We filtered 700-1500 ml of each sample on pre-combusted (550 °C, 12 h) 47-mm Whatman™ GF/F filters, until clogging occurred. The filters were dried for 24 h at 45 °C and frozen. To encapsulate the seston material in tin capsules, a 36-mm diameter punch was used to subsample 81 % of the seston. The encapsulated samples were then frozen at -20 °C for up to 3 months before further processing.

Zooplankton were normally sampled between 12:00-20:00 hours with a 25-L Schindler-Patalas plankton trap fitted with a 100-μm net. Repetitive casts were made until between 25-200 L of water were collected from each depth. Higher volumes were collected in the epilimnion because zooplankton were sparse there during the day. The 100-μm net may have retained a small amount of filamentous phytoplankton in addition to the zooplankton, though this type of phytoplankton is scarce in Laguna El Tejo (Morata *et al.*, 2003; our data). The zooplankton samples were filtered on pre-combusted, 25-mm GF/F filters, placed in Eppendorf capsules, dried 24-48 hours at 45 °C and then encapsulated for isotopic processing.

Sedimenting material was collected in four traps placed at each station. The traps were 12-cm diameter, and 50-cm long plastic cylinders. Prior to deployment, these were filled with deionized water containing 5 g/L reagent grade NaCl in order to create a density gradient and harsh conditions to prevent lake water and non-sedimenting plankton, respectively, from entering the trap during deployment. We then injected 120 ml of 2 % formaldehyde and 1 % of sodium tetraborate into the bottom of each trap to preserve sedimenting material. Formaldehyde has minimal effects on measurements of isotopic enrichment (Bicknell *et al.*, 2011). The traps were then suspended in the anoxic layer with the trap mouth at 21-m for 1-4 weeks. When a trap was brought to the surface, we homogenized the contents, measured the volume, and a 60-100 ml subsample was filtered on pre-combusted 47-mm GF/F filters. The filters were then dried and processed in the same manner as the seston samples.

^{15}N analyses and calculations

Nitrogen mass and isotopic composition were determined with a Europa Scientific ANCA 2020 mass spectrometer. To estimate the flux of ^{15}N into different compartments we calculated the increment in the total amount of ^{15}N above those at the beginning of the experiment, under the assumption of stable conditions over the 28-day period. We did not collect base-line conditions for the δ^{15}N of sedimenting material. Consequently, we used the depth-weighted mean δ^{15}N of seston in the water column prior to the injection ($^{+}\delta$ 4.8) as the background level for sedimenting material. If epilimnetic seston, with lower δ^{15}N levels ($^{+}\delta$1.4 - 1.9), sedimented more than that in the metalimnion, our calculation would have underestimated the amount of material transported to the sediments.

The proportions of epilimnetic (f_1) and metalimnetic seston (f_2) consumed by zooplankton was estimated with a simple mixing model (Phillips, 2012):

$$f_1 = \frac{(\delta^{15}N_{mix} - \delta^{15}N_{tr}) - \delta^{15}N_2}{\delta^{15}N_1 - \delta^{15}N_2}$$

$$f_2 = 1 - f_1$$

Where:

$\delta^{15}N_{mix}$ = Mean incremental isotopic enrichment above background in zooplankton from a given strata on day 28 of the experiment

$\delta^{15}N_{tr}$ = Mean trophic enrichment factor of zooplankton above seston prior to tracer addition (δ 8.2)

$\delta^{15}N_1$ = Mean incremental enrichment in seston from epilimnion (3 and 8 m)

$\delta^{15}N_2$ = Mean incremental enrichment in seston from metalimnion (14 -18 m)

Rhodamine fluorescence was measured on water samples collected with the Ruttner bottle used for ^{15}N-seston samples that were frozen and subsequently filtered (GF/F) and analyzed with a Hitachi F4500 fluorescence spectrophotometer set with excitation and emission wavelengths set at 540 and 576 nm, respectively, and with 5 nm slit widths. Because rhodamine degrades when exposed to light, we conducted an experiment that allowed us to correct for this degradation in the lake. Rhodamine WT was dissolved in deionized water and placed in Pyrex bottles and then either exposed directly to the sun for 120 hours or held in the dark. In the dark bottles there was no significant degradation during the experiment ($p = 0.19$). Light measurements were measured concurrently and the relationship between accumulated light (PAR, in Einsteins = mol Photon m^{-2} s^{-1}) and rhodamine fluorescence in the bottles was established:

$$\% \text{ of initial} = 4.554 + -0.00256 \ln (\text{Einsteins}),\ r^2 = 0.91$$

A model of rhodamine degradation in the lake was established using a mean vertical extinction coefficient for PAR of 0.201/m, and assuming 14 h of full sunlight each day:

$$\% \text{ of initial rhodamine} = e^{(4.55 - 0.00256 * E)}$$

where E = cumulative Einsteins over a given number of days

At depths of 10, 14 and 16 m this resulted in a correction factors of 0.54, 0.80 and 0.82, respectively, after the 28-day main experiment in the lake. The model, however, did not correct for differential rhodamine degradation with shifting wavelengths at different depths, so it is only approximate.

Eddy diffusivity of the rhodamine (K_z) and “biodiffusivity” (K_b) of the ^{15}N were calculated as follows (Quay *et al.*, 1980):

$$K_{(z \text{ or } b)} = \frac{\sigma_t{}^2 - \sigma_o{}^2}{2\,(t - t_0)}$$

Where:

t = initial (t_0) and time since injection (σ_t; seconds)

σ_t = mean square distance (m)
= ½ distance between depth at which the rhodamine or ^{15}N was 0.1 x the maximum concentration

σ_o = 0.5 m, tracer injection thickness

Initial mean square distances were calculated with data from the mean rhodamine fluorescence profiles recorded ~5 hours after the injection on a Wetstar® (Wetlabs, Inc.) in situ fluorometer mounted to a Seabird 19 CTD. Fluorescence recorded 7-hours previously was subtracted from the rhodamine signal because phycoerythrin in cyanobacteria fluoresced slightly in the rhodamine spectra. Because rhodamine and ^{15}N were injected concurrently, this fluorescence measurement should provide an accurate picture of the relative dispersion of the tracers at the injection depth. Subsequent measurements of the tracers were made from the seston or rhodamine samples collected from the Ruttner bottle. Because the Ruttner bottle was 0.5-m long, and because we sampled only at 1.5-3 m intervals, our estimates of K_z and K_b are not precise. However, the *relative magnitudes* of the two diffusivity estimates should not have been influenced by the sampling design.

Physical and chemical measurements

In addition to the Seabird CTD profiles, we collected additional measurements at one station in the lake on each sample date. *In situ* temperature, conductivity and oxygen profiles were made with WTW meters (LF 191 and WTW Oxi91). Light penetration (photosynthetically active radiation, 400-700 nm) in the lake was measured by a 4π scalar irradiance sensor (Li-Cor). Chemical analyses were performed according to standardized methods (Golterman, 1978; APHA, 1992) on three dates (17 Sep, 30 Sep, 15 Oct). Ammonia (detection limit (DL) = 2.8 µg N/L) was measured by the indophenol blue method. Nitrate plus nitrite (DL = 0.3 µg N/L) was measured as nitrite after reduction of nitrate by a cadmium-copper couple in an alkaline solution, and colorimetric determination with sulfanilamide and N-(1-naphtyl) ethylenediamine dihydrochloride. Particulate nitrogen (PN) from samples retained on GF/F filters was analyzed with the mass spectrometer as described above. Soluble reactive phosphorus (SRP; DL = 0.9 µg P/L) was measured by the phosphomolybdic acid method with ascorbic acid. Total phosphorus (DL = 0.9 µg P/L) was determined as orthophosphate after persulfuric acid digestion of the sample for 2 h at 135 ºC.

Plankton measurements

Phytoplankton were identified and counted from each of the sampling depths for samples collected on 17 Sept., 2 Oct., and 15 Oct. Phytoplankton composition was relatively stable on these three dates, and we have consequently presented data from only the midpoint of the experiment (2 Oct.) when the biovolume of each taxon were also estimated. Eukaryotic phytoplankton counts were made using the Utermöhl sedimentation method (Uthermöhl, 1958) in an Olympus inverted microscope at 200-1000 magnification. Species were identified following the taxonomic keys described in Sendra *et al.* (2019). Biovolume estimates were made by measuring several cells of each phytoplanktonic species, then geometric shapes were used for calculations (Rott, 1981). Picocyanobacterial counts were performed by epifluorescence microscopy on a Zeiss III epifluorescence microscope as described by Camacho *et al.* (2003c). Chlorophyll *a* was measured on each sampling date after filtration of the samples

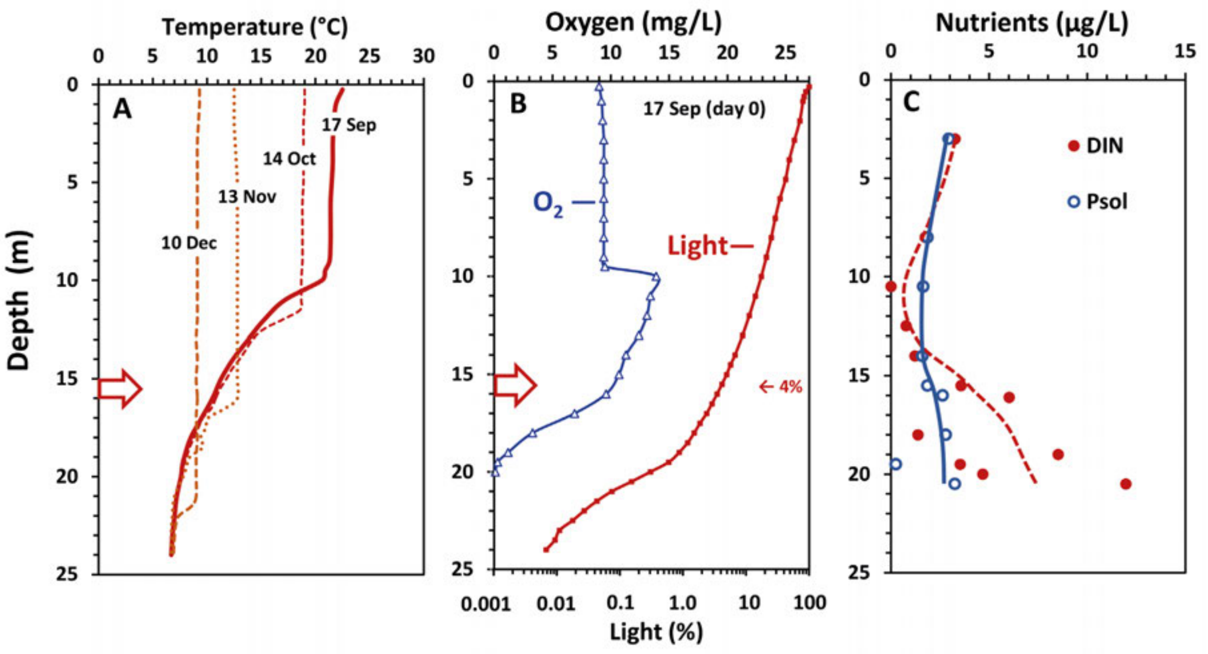

Figure 2. A. Profiles of temperature on five dates during the experiment in Laguna El Tejo. B. Profiles of oxygen concentration and light intensity at the start of the experiment on 17 September. The arrows on the y axes show the depth that ^{15}N and rhodamine were injected into the deep chlorophyll layer. C. Mean dissolved inorganic nitrogen (DIN) and soluble reactive phosphorus (SRP) measured on three dates during the 28-day experiment. *A. Perfiles verticales de temperatura medidos en cinco fechas diferentes durante el experimento realizado en la Laguna del Tejo. B. Perfiles verticales de la concentración de oxígeno y la radiación luminosa medidos al principio del experimento, el 17 de septiembre. Las flechas muestran la profundidad en la que se inyectó el* ^{15}*N y la rodamina en la profundidad donde estaba localizado el máximo profundo de clorofila. C. Concentraciones promedio de nitrógeno orgánico disuelto (DIN) y fósforo reactivo soluble (SRP) en tres fechas a lo largo de los 28 primeros días del experimento.*

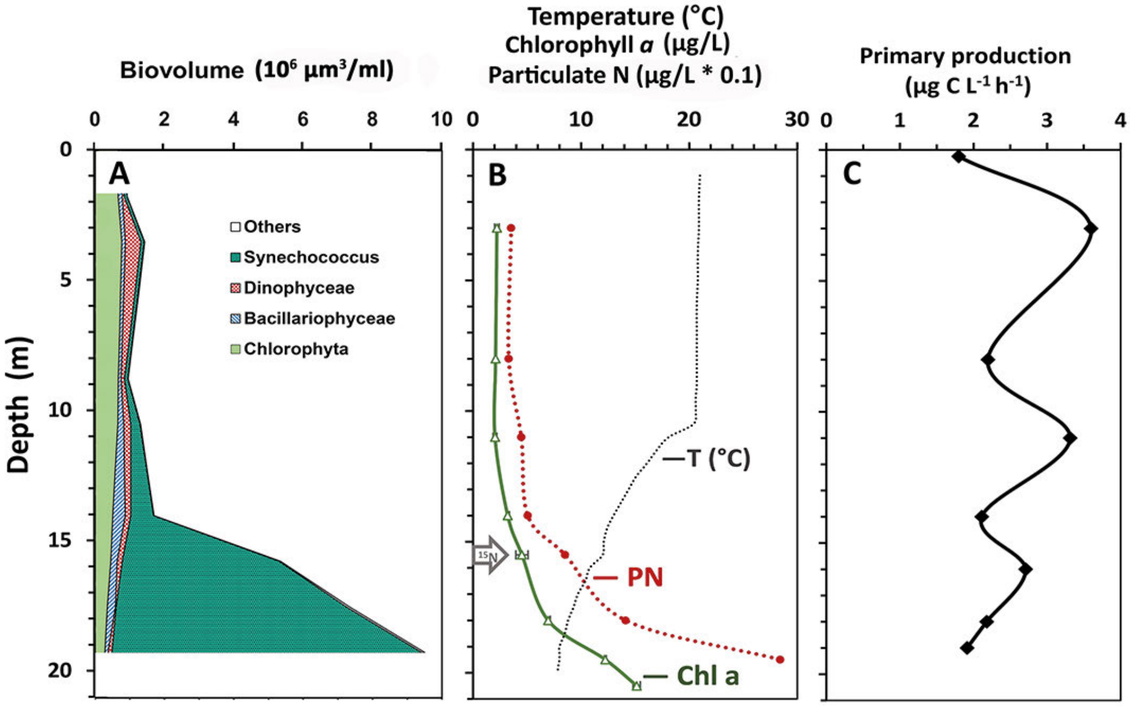

Figure 3. A. Biomass composition of phytoplankton groups in the water column of Laguna El Tejo on Day 15 (2nd-October) of the experiment. B. Chlorophyll *a*, particulate nitrogen concentrations (PN) and temperature in the water column on the Day 13 (30th Sept.). C. Plankton primary production profile on Day 13. *A. Composición (en biomasa) de los grupos principales de fitoplancton a lo largo de la columna de agua de la Laguna del Tejo en el día 15 del experimento (2 de octubre). B. Concentraciones de clorofila-*a *y de nitrógeno particulado (PN) y temperatura en el día 13 del experimento (30 de septiembre). C. Perfil vertical de la producción primaria en el día 13 del experimento.*

through a GF/F glass fiber filter, and subsequent extraction with acetone: DMSO, following the procedures described in Picazo *et al.* (2013). Carbon photosynthetic assimilation was measured *in situ* on 30 Sept. using the ^{14}C technique (Steeman-Nielsen, 1952) following the procedure described by Camacho & Vicente (1998). Samples were directly placed in 60-ml tissue culture bottles (Nunclon, Intermed) and kept in the dark during manipulation. Two clear and a dark bottle were used for each depth. 4 µCi of $NaH^{14}CO_3$ was added to each bottle and they were then incubated at depth for 4 hours. Inorganic carbon estimates were made using measures of alkalinity, pH, temperature, and ionic strength.

Midway through the study (30 Sept. - 1 Oct.) we measured diel vertical migration of the zooplankton at one station. At midday and midnight vertical profiles were made with the Schindler-Patalas trap and the samples preserved in 4 % formalin for subsequent identification and biomass estimates. Entire samples were counted on an inverted microscope at 100 X. For crustacean species, > 100 individuals were measured to estimate their biomass utilizing length–weight regressions (Lawrence *et al.*, 1987). To characterize the fraction of smaller zooplankton (30-100 µm, mainly rotifers) we re-filtered the 1L 100-µm filtrate with a 30-µm Nitex net. These samples were preserved and counted using an inverted microscope at 100–400 magnification. Estimates of the biomass of rotifers were made following Ruttner-Kolisko (1997).

Four-five minnows were sampled with a seine at the start of the experiment and after 15, 28 and 57 d. These were dried, ground and subsamples were analyzed for ^{15}N enrichment as described for zooplankton.

RESULTS

Limnological characteristics

The experiment was conducted during the fall, and consequently the epilimnion cooled and deepened from mid-Sep until December. However, the change was relatively minor during the main experiment, from 17 Sep. to 14 Oct. (Fig. 2A). Oxygen concentration peaked at the top of the metalimnion, where the density gradient minimized upwards diffusion of the photosynthetically-produced oxygen, and declined to 0 mg/L by 20 m. Secchi disk visibilities were 7.2 m and 7.4 m, respectively, at the start and end of the 28-day experiment. Light levels declined nearly exponentially through the epilimnion and upper metalimnion but dropped rapidly in the hypolimnion where the deep chlorophyll layer was well developed (Fig. 2B). Light levels were 4 % of surface intensities at the ^{15}N injection point. The depth of 1 % light intensity changed from 18.7 m at the start of the experiment to 17.9 m on day 28. The 1 % light level was near the peak of the Synechococcus layer (see below). Dissolved inorganic nutrients were low in the epilimnion, dropped lower at the top of the metalimnion, and increased below 15 m in the deep chlorophyll layer (Fig. 2C). On most dates total P in the epilimnion was near the level of detection (0.9 µg/L), increased to 3 µg/L in the upper DCL and reach 16 µg/L at 20 m. The spring water entering El Tejo that was sampled on three dates had mean concentrations of 3.6 ± 3.2 µg N/L NH_4^+, 760 ± 526 µg N/L $NO_3^+NO_2$ and 1.0 ± 0.7 µg/L soluble P.

Algal composition in Laguna El Tejo varied markedly with depth (Fig. 3A). In the epilimnion, green algae (*Cosmarium laeve*, *C. abbreviatum*, and others) were co-dominants with a large dinoflagellate (*Peridinium* sp.), a small population of diatoms (*Fragilaria ulna*, *Cyclotella radiosa*) and *Synechococcus*. In the upper part of the deep chlorophyll layer (11-14 m), green algae (*C. abbreviatum*, *C. laeve* and others) and diatoms (*C. radiosa*) were abundant, along with dinoflagellates and increasing populations of *Synechococcus* sp.. A very small population of the cyanobacterium *Pseudanabaena* sp. was found in the epilimnion and metalimnion on one date. At 15-16 m in the deep chlorophyll layer, green algae (*Chlorella vulgaris*; *Cosmarium* spp.), diatoms (*C. radiosa*) and dinoflagellates remained abundant, but were overshadowed by increasing densities of *Synechococcus* which represented 85 % of the phytoplankton biovolume. At the anoxic interface at 19.5 m, the biomass of green algae, diatoms and dinoflagellates decreased and cryptophytes (*Cryptomonas erosa*, *C. phaseolus*) and euglenophytes (*Euglena agilis*) increased moderately. At 19.5 m, however, *Synechococcus* densities reached 4-5 x 10^6 cells/ml, and they represented 95 % of the autotrophic plankton biovolume. At 19.5 m estimated phytoplankton biovolume was 10-times higher than in the epilimnion.

All three biomass metrics (algal biovolume, seston N and Chl. *a*) had similar depth profiles, suggesting that most of the seston was comprised of phytoplankton. However, the seston N metric also would have included detrital material. The changes in phytoplankton biovolume with depth were paralleled by increases in particulate nitrogen, which increased from 34 µg/L in the epilimnion, to 85 µg/L at the tracer injection depth, to 284 µg/L at 19.5 m (Fig. 2B). Extracted chlorophyll *a* concentrations also followed the same pattern as seston N, increasing some in the upper metalimnion and then markedly in the strata where *Synechococcus* predominated (Fig. 3B). The chlorophyll profile changed little throughout the experiment, with the exception that the low epilimnetic concentrations extended to 12.5 m by day 28, coincident with the increased mixing depth (Fig. 2A).

Photosynthesis, on the one day it was measured, had an irregular pattern with one peak in the epilimnion, a second one at the top of the deep chlorophyll layer, and moderate production throughout the *Synechococcus* layer (Fig. 3C). At 19 m, where the light intensity was only 12 µE m^{-2} s^{-1}, primary production equaled that of phytoplankton at the surface. The production at the top of the deep chlorophyll layer was accompanied by a peak in oxygen concentrations (Fig. 2B).

The deep layers of the lake dominated algal biomass metrics, and to a lesser degree, primary production. Only 21-38 % of the algal biovolume, chlorophyll and particulate nitrogen

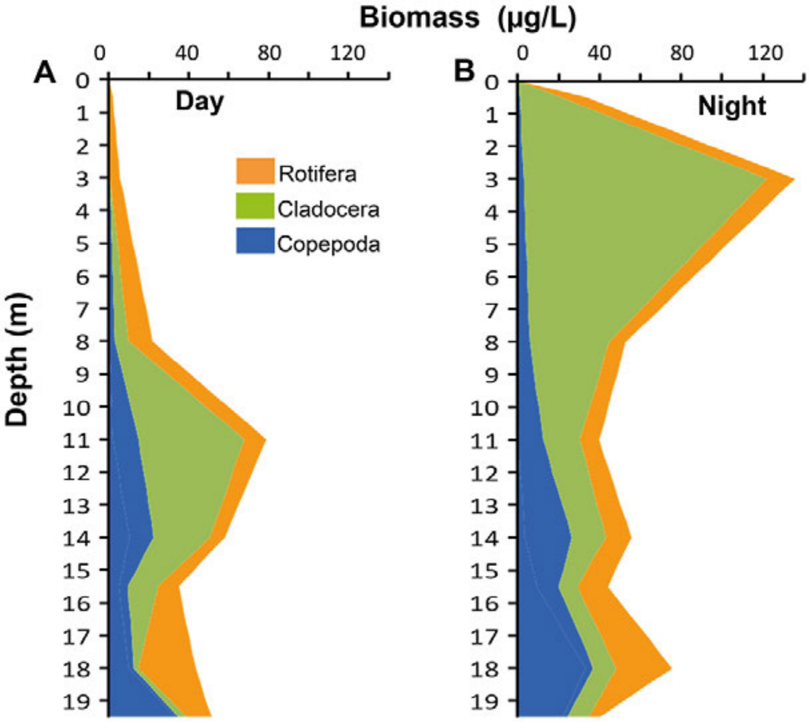

Figure 4. Dry biomass of different zooplankton groups at different depths of Laguna El Tejo (A) during the day and (B) night on 1 Oct. *A. Biomasa (peso seco) de los diferentes grupos de zooplancton en el perfil vertical de la Laguna del Tejo (A) durante el día y (B) por la noche, del día 1 de octubre.*

biomass occurred in the epilimnion (0-10.5 m), whereas 58 % of the primary production occurred there (Fig. 3). An additional 10-31 % of biomass metrics occurred in the 10.5-14 m strata above the *Synechococcus*-dominated zone, and 19 % of primary production occurred there. The Synechococcus zone (> 14 m), accounted for 48-70 % of the biomass metrics, and 23 % of the primary production.

Zooplankton biomass in the lake was dominated by the herbivores *Diaphanosoma brachyurum* and *Tropocylops prasinus* and by the predaceous copepod, *Cyclops abyssorum* (Fig. 4). For both copepods, larval stages (which are mostly herbivores) accounted for a 60 % of their biomass. During the day, the grazers (*D. brachyurum* and *T. prasinus*) occurred primarily in the upper part of the deep chlorophyll layer (11-14 m) at temperatures between 13° and 19 °C. Their densities declined at 15.5 and particularly at 18 and 19.5 m where *Synechococcus* increased markedly. At night, the *Diaphanosoma* increased in the epilimnion, primarily as a result of diel horizontal migration (Armengol *et al.*, 2012). Rotifers represented around 25 % of the biomass in the water column and *Anuraeopsis fissa*, a species that frequently peaks in the hypolimnion of stratified lakes (Miracle & Armengol, 1995), were particularly abundant in the *Synechococcus* zone. Nitrogen concentrations of the zooplankton followed the same profile as the zooplankton biovolume estimates, but concentrations were less than 2 % of that in the seston (data not shown).

^{15}N enrichment of the community

Prior to the addition of tracers, the natural ^{15}N enrichments of phytoplankton and zooplankton community increased with depth (Fig. 5). δ^{15}N levels for seston increased from $^{+}$1 in the epilimnion to $^{+}$6 in the hypolimnion. Zooplankton δ^{15}N levels increased in a similar manner,

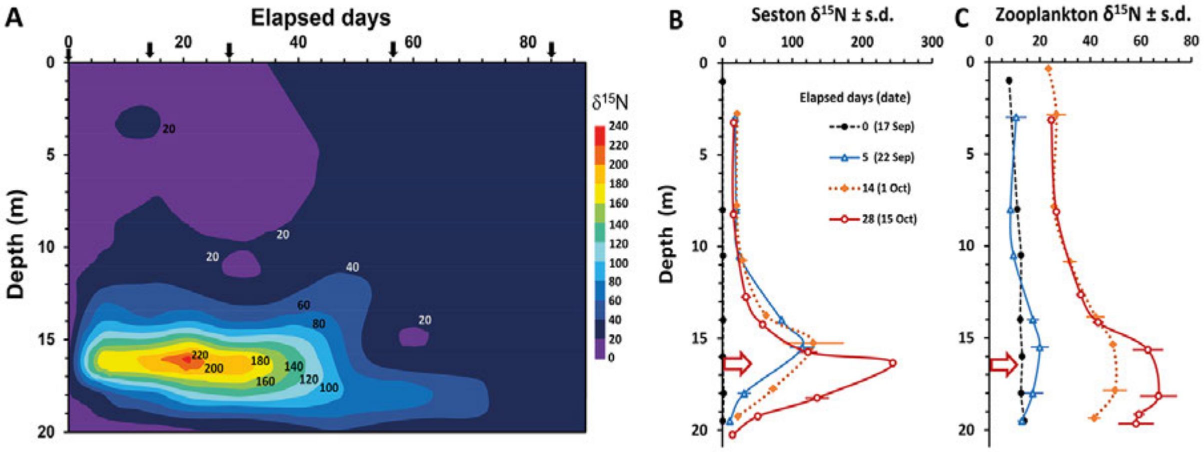

Figure 5. A. Isopleths showing ^{15}N enrichment in the seston for the first 85 days of the experiment. Arrows show the dates where isotopic enrichment was measured. B. Depth profiles of ^{15}N enrichment of the seston on four dates. C. ^{15}N enrichment of the zooplankton on four dates. *A. Isolíneas del enriquecimiento en ^{15}N del seston en los primeros 85 días del experimento. Las flechas muestran la fecha y profundidad en la que se inyectó el trazador. B. Perfiles verticales del enriquecimiento en ^{15}N del seston en cuatro fechas durante el estudio. C. Enriquecimiento en ^{15}N del zooplancton en cuatro fechas durante el estudio.*

from +8 near the surface to +14 at 19.5 m. Zooplankton, on average, had a trophic enrichment factor (+$\delta^{15}N$) 8.2 units above the seston.

After the addition of $^{15}NH_4^+$, the seston pool became labeled with the tracer, particularly near the depth of the injection (Fig. 5A-B). Within 5 days $\delta^{15}N$ levels at 15.5 m increased to +110 and continued to increase until day 30. On day 30 we sampled one additional depth (16.1 m) and found even higher labeling there. Surprisingly, by day 5 ^{15}N labeling also increased to near +20 in the epilimnion, likely the result of contamination when our injection tube with pulled up from 15.5 m without first flushing it. Epilimnetic seston labeling did not increase subsequently during the main 28-day experiment. By 13 Nov. when winter mixing had begun, seston enrichment was near +30 throughout the mixed layer that extended to 16 m, and by 10 Dec. the lake was nearly isothermal (Fig. 2A) and the isotope had mixed throughout the water column, enriching the seston to +33 (Fig. 5A).

Zooplankton also became enriched with ^{15}N, particularly in the deep chlorophyll layer (Fig. 5C). After 5 days, enrichment was limited to zooplankton in the deep chlorophyll layer, but by days 14 and 30, zooplankton became enriched throughout the water column. In the epilimnion, enrichment averaged +26, higher than the seston in that layer, but consistent with the trophic enrichment factor noted prior to the tracer addition, indicating that the epilimnetic zooplankton were feeding little in the deep chlorophyll layer. In the deep chlorophyll layer, zooplankton enrichments peaked at +50 (day 14) and +67 (day 30) at 18 m.

The mixing model analysis of zooplankton diets also indicated that zooplankton collected in the epilimnion (3-8 m) were consuming almost entirely seston from that layer (Fig. 6; 3-8 m). At the top of the metalimnion (12.5-14 m), the model estimated that 14 % of the diet was from the deep chlorophyll layer, and in the *Synechococcus* layer (15.5-19 m), 33 % of the zooplankton diets were from that layer, with 64 % estimated to have been from epilimnetic seston.

^{15}N injected into the deep chlorophyll layer and incorporated in the seston and zooplankton moved in the water column 10 times faster than the rhodamine (Fig. 7). Vertical bio-diffusivity for the ^{15}N seston was 1.24 * 10^{-6} m^2/s and the diffusivity of rhodamine was only 0.12 * 10^{-6} m^2/s.

$\delta^{15}N$ increased slightly in fish from +10.6 ± 0.2 at the start, to +12.0 ± 1.7 on day 28 (*p* =

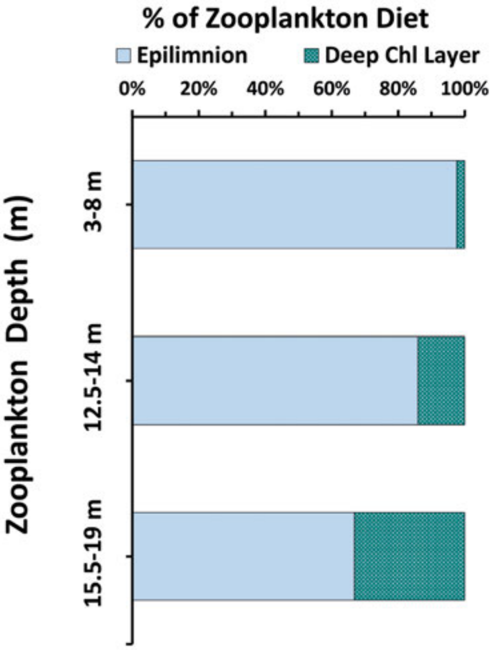

Figure 6. Mixing-model estimates of the diet proportions of macrozooplankton from different depths consumed from the epilimnion, and from the deep chlorophyll layer. Because the model assumes that the zooplankton had reached isotopic equilibrium, the diet proportion from the deep chlorophyll layer may be underestimate. Data are for day 28 of the experiment. *Estimaciones, mediante un modelo mixto, de la proporción de la dieta del macrozooplancton de diferentes profundidades consumida en el epilimnion y en el máximo profundo de clorofila. Dado que el modelo asume que el zooplancton ha alcanzado su equilibrio isotópico, es posible que la proporción de la dieta ingerida en el máximo profundo haya sido subestimada. Los datos corresponden al día 28 del experimento.*

0.09), but then decreased to +9.9 ± 1.3 in mid-November (p = 0.63), indicating that fish benefited little from the deep chlorophyll layer.

An analysis of the ^{15}N distribution on day 28 indicated that 55 % of the injected tracer was accounted for. The seston in the metalimnion (11.5-18m) accounted for over 70 % of this, indicating relatively little movement into the epilimnion and hypolimnion (Fig. 8). Zooplankton incorporated only 1 % of the ^{15}N that was added and 8 % was found in the sediment traps. Mean sedimentation rates for the first and second two-week periods were 26.7 and 21.8 mg N m^2/d, but these were not significantly different (2-tailed t-test, p = 0.16). Average particulate nitrogen levels in the 20-m water column above the traps was 1400 mg N/m^2, yielding an average daily loss rate of 1.6 %/d. In contrast, the sedimentation loss rate for ^{15}N was eight times lower: only 0.2 %/d.

The year-long analysis of ^{15}N in the epilimnion of El Tejo showed that the tracer declined exponentially after the lake mixed (Fig. 9). After 335 days post-injection the epilimnetic seston remained +δ 5.6 above background enrichment. The decline was slow, with an estimated tracer half-life of 170 d.

DISCUSSION

The sampling demonstrated that much of the algal production (42 %) and biovolume (78 %) in this karst lake occurred in the metalimnion and hypolimnion. This has routinely been found in oligotrophic lakes and oceans (Giling *et al.*, 2017) and even in many mesotrophic systems

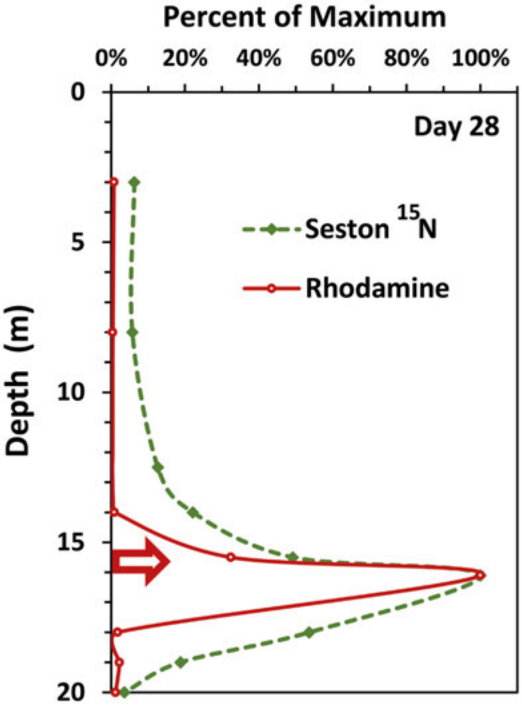

Figure 7. Distribution of rhodamine and ^{15}N-labeled seston on day 28 of the experiment. The arrow shows the injection depth of the tracers. *Distribución de la rodamina y del seston marcado con ^{15}N en el día 28 del experimento. La flecha muestra la profundidad de inyección de los trazadores.*

(Williamson *et al.*, 1996). In Lake El Tejo, phytoplankton biomass and chlorophyll levels began to increase in the top of the metalimnion, but the large increase was deep in the water column where a cyanobacterial *Synechococcus* population developed at a light intensity near 4 % of surface radiation. Although DCLs frequently development near the top of the metalimnion, Leach *et al.* (2018) found that 33 % of lakes they analyzed with DCM had chlorophyll peaks below the metalimnion. They also found that the median light level for development of the DCM was 4.8 %, similar to that found in El Tejo, whereas DCL are common at up to 1 % PAR depths (Camacho, 2006; Scofield *et al.*, 2020).

Deep chlorophyll layers are typically composed of motile algal species and/or neutrally buoyant cyanobacteria with gas vacuoles or ballast substances (Richardson & Cullen, 1995; Camacho *et al.*, 2000; Leach *et al.*, 2018). The upper part of the DCL in El Tejo was due primarily to a diatom (*Cyclotella radiosa*), but the large increase in chlorophyll and algal biomass was due to *Synechococcus*, which has a diameter near 2 µm and a low cellular density, thus having negligible sinking rates (Camacho *et al.*, 2003b). The large peak in *Synechococcus* is similar to that of the nearby Lake La Cruz (Camacho *et al.*, 2003c) where active growth occurs in the deeper part of the metalimnion, and cell densities reach nearly 10^7 cells/ml. Light-harvesting and growth of these picocyanobacterial is possible in the deep layers because they contain phycoerythrin (Camacho *et al.*, 1996; Camacho *et al.*, 2001; Camacho *et al.*, 2003b). Consequently, they can thrive at depths where nutrient availability is higher than in the surface layers (Camacho, 2006). Moreover, our primary production determinations showed a peak of carbon assimilation coinciding with the deeper DCL formed almost exclusively by *Synechococcus*, which evidenced *in situ* photosynthetic activity even near the oxic-anoxic interface. However, its activity was much lower per biomass unit than the upper (subsurface and metalimnion) primary production peaks, which yield higher specific productivity (production/biomass). Earlier experiments demonstrated that even though they have phycoerythrin, the *Synechococcus* are nevertheless light

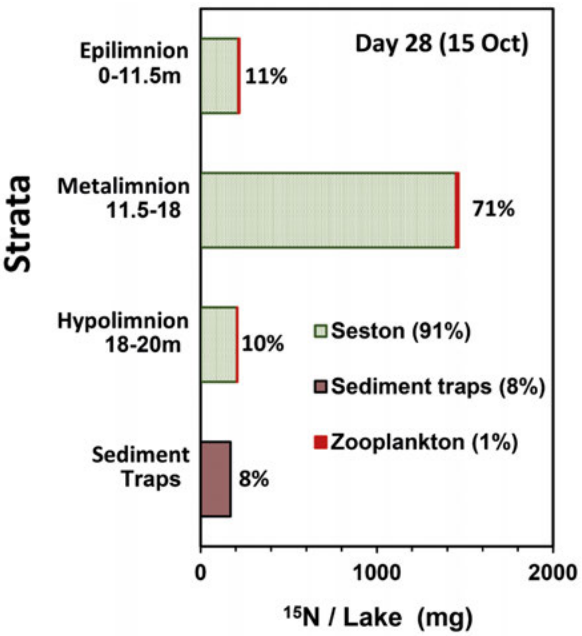

Figure 8. Estimated distribution of the ^{15}N recovered at the end of the experiment (day 28). *Distribución estimada del ^{15}N recuperado al final del experimento (día 28).*

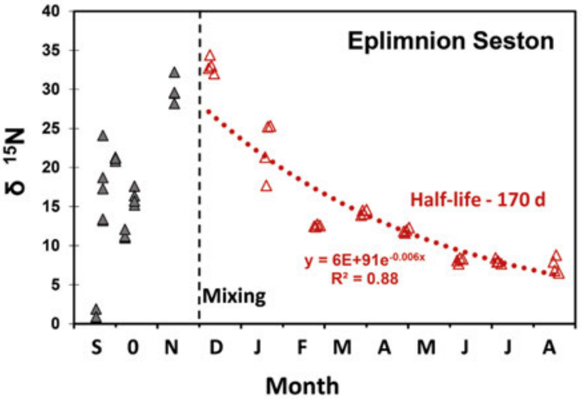

Figure 9. Enrichments of ^{15}N in the seston of Laguna El Tejo over one year. The solid grey triangles show isotopic enrichment of seston prior to lake mixing. The red open triangles show the decline in enrichments after the lake had mixed nearly to the bottom. *Enriquecimiento en ^{15}N del seston en la Laguna del Tejo un año después de la inyección. Los triángulos grises muestran el enriquecimiento del seston antes de la mezcla del lago. Los triángulos rojos vacíos muestran la caída del enriquecimiento tras la mezcla vertical del lago.*

limited, but they take advantage of the higher nutrient availability in these deeper layers to support *in situ* growth (Camacho *et al.*, 2003b). Negligible sinking rates and low grazing pressure in the cold hypolimnion minimize population losses of *Synechococcus* in the DCL, allowing high biomass accumulation, despite low growth rates (Camacho *et al.*, 2003a, 2003b).

The seston in El Tejo became heavily labeled with ^{15}N within five days of the addition, primarily in the DCL at 15.5 m. On day 28 of the experiment we sampled ^{15}N slightly deeper (16.1 m) because rhodamine profiles measured with the WetStar fluorometer mounted on the Seabird CTD indicated the peak was there, and we found that isotopic labeling was nearly double that of the seston at 15.5 m. The higher enrichment at 16.1 m was likely due to either a slight measurement error with our injection line, or to a higher density of the injected tracers that could have carried them below their injection point. The seston in the epilimnion was also enriched to δ+18 within five days but showed no additional enrichment during the main 28-day experiment. Wilkinson *et al.* (2014) injected ^{13}C tracer into the metalimnion of a similar-sized lake and found no enrichment of seston in the epilimnion until after 20 days. Consequently, we believe the epilimnetic enrichment in El Tejo was primarily due to some spillage from our raft, or from accidental addition when the injection tube was raised from depth without first flushing it. Rhodamine was not measured on the first sampling date following the injection, but on day 16 of the experiment average epilimnetic rhodamine was 2.6 % of the concentration at 15.5 m, indicating that some tracer contamination of the epilimnion occurred during the injection.

After 28 days we were only able to account for 55 % of the ^{15}N tracer. Other pools that we did not measure that could account for this missing tracer are: (1) ^{15}N uptake by periphyton or benthic bacteria, which can be significant in oligotrophic lakes (Brothers *et al.*, 2016; Vadeboncoeur & Power, 2017); (2) denitrification, which could have been appreciable since the $^{15}NH_4^+$ injected could have easily been nitrified and then denitrified at the interface of the oxic-anoxic zone at 19-20 m (e.g. Brezonik, 2013; Castellano-Hinojosa *et al.*, 2017); (3) $^{15}NH_4^+$ in the injection strata which was not taken up by the plankton and tracer transformed into dissolved inorganic nitrogen, neither of which were analyzed for; (4) tracer leaving the lake via groundwater, and; (5) errors in our measurements of the other pools. Because we didn't have a lake-wide estimate of fish abundance or biomass we could not calculate the amount of ^{15}N moving into this pool, but the insignificant ^{15}N enrichment in their tissue, and the normally small contribution of fish to the biomass of the biota (Wurtsbaugh, 2007), suggests they were not an important pool for the tracer.

Only 8 % of the recovered tracer had fallen into the sediment traps. This low amount was not surprising given that: (1) much of the tracer was likely incorporated into *Synechococcus*, with very low sedimentation rates (Camacho *et al.*, 2003b; Camacho, 2006) and; (2) macrozooplankton grazing in the DCL was likely very low, with consequent low export of fecal material that can account for significant losses of particulate matter from the water column of lakes and oceans (Pilati & Wurtsbaugh, 2003; Turner, 2015; Maszczyk & Wurtsbaugh, 2017). Additionally, grazing on the abundant *Synechococcus* was likely done by the abundant rotifer, *Anuraeopsis fissa*, that graze bacteria and picoplankton efficiently (Ooms-Wilms, 1997; Twiss *et al.*, 2012). This, however, would not cause vertical movements of the ^{15}N, since rotifers have limited vertical migrations (Armengol & Miracle, 2000) and they do not produce dense (settling) excreta such as the fecal pellets of copepods. The sedimentation rate of the ^{15}N was eight times lower than the sedimentation rate of unlabeled N, suggesting that much of the settling material originated above the heavily labeled *Synechococcus* layer.

The comparison of the final rhodamine and ^{15}N profiles in the DCL indicated that biotic processes were 10X more important for tracer movement than the physical process of vertical eddy diffusion. One obvious reason for the faster movement of ^{15}N was sedimentation of the phytoplankton and excreta of zooplankton that fed in this layer. Although ^{15}N sedimentation rates were low, they likely contributed to the downward spread of the tracer. The greater upward movement of the tracer could have been due to mobile flagellates, or to ^{15}N in the excreta of zooplankton that had some vertical movement in the water column (Fig. 4; Armengol *et al.*, 2012). The greater dispersion of the ^{15}N must be viewed in the context of the low diffusivity of the rhodamine tracer, $0.12 * 10^{-6}$ m^2/s, which is near that for molecular diffusion (Kalff, 2002). This low diffusivity is consistent with the very small fetch of El Tejo (140 m) and the surrounding 20-50 m cliffs that protect the lake from winds. Based on the surface area of El Tejo, the equation of Maiss *et al.* (1994) predicts a rhodamine diffusivity of 0.435×10^{-6} m^2/s, somewhat higher than what we observed, but consistent with the wind-protected lake surface. The higher diffusivity for ^{15}N via biotic processes (biodiffusivity) is important because researchers often address the upward vertical movement of nutrients as a strict consequence of the physical mixing processes, and thus they are likely underestimating the true movement of nutrients. Although biodiffusivity has been studied extensively to address solute transport from the sediments (Matisoff, 1996; Kristensen *et al.*, 2012), its importance in the water column has focused primarily on how zooplankton influence physical mixing (Simoncelli *et al.*, 2017; but see Pilati and Wurtsbaugh (2012); Houghton *et al.*, 2018).

After fall mixing dispersed ^{15}N throughout the water column, we continued to sample the epilimnetic seston until the following August, allowing us to estimate a half-life for this tracer of 170 d. Given that there are no surface inflows to El Tejo, and that the lake lost depth during our study, we expect that groundwater exchange with the lake was low during the study thus providing minimal flushing. However, we do not have a water residence time to compare with the ^{15}N tracer, because we could not use the long-term loss rate of rhodamine due to unknown spectrally-controlled photo-bleaching. Nevertheless, the data demonstrate that denitrification and sedimentation loss of nitrogen was low in this lake.

Our data indicates that the production in the DCL was only moderately important as a food source for crustacean zooplankton, despite the amount of primary production and particularly algal biomass in this layer. After 28 days, only 1 % of the recovered ^{15}N was found in the zooplankton. This was somewhat expected, given that only 1.8 % of N in the plankton was in the crustacean zooplankton. The mixing model also indicated that the DCL was relatively unimportant as a food resource for the zooplankton: only 2 % of the food consumed by epilimnetic zooplankton and 14-33 % by macrozooplankton captured in the DCL was from the deep layer. Many crustacean zooplankton can feed on *Synechococcus* (Lampert *et al.*, 1986), so the low values were unexpected. We did not, however, measure isotopic enrichment of rotifers, as nearly all of them would have passed through the 100-µm mesh we used to separate seston and zooplankton. Rotifers can feed heavily on picoplankton such as the *Synechococcus* in the DCL of El Tejo (Twiss *et al.*, 2012), so it is likely we underestimated the transfer to the zooplankton. Additionally, the zooplankton sampled on day 28 may not have come into full equilibrium with ^{15}N tracer in the seston and this also could have caused us to underestimate the importance of the DCL. However, in a ^{13}C metalimnetic enrichment experiment similar to ours (Wilkinson *et al.*, 2014) zooplankton isotopic labeling was complete within 30 days, suggesting that the plankton in El Tejo may have been close to equilibrium.

Based on their ^{13}C experiment Wilkinson *et al.* (2014) inferred that the DCL contributed only minimally to the crustacean zooplankton diet, and Sanful *et al.* (2017) studying natural populations, reached a similar conclusion. However, Francis *et al.* (2011), using natural-abundance isotopic analyses, concluded that the DCL was a very important food source in oligotrophic lakes. Others have found conflicting evidence of the food value of seston in the DCL (Williamson *et al.*, 1996; Cole *et al.*, 2002). Cold temperatures at the depth of the DCL in El Tejo (8-13 °C) and other lakes should also reduce zooplankton grazing in this stratum (Dawidowicz & Loose, 1992; Lampert *et al.*, 2003). Much of the literature on the importance of the DCL for zooplankton has focused on diel vertical migration (DVM), and the consumption of deep-chlorophyll phytoplankton during the day when zooplankton migrate into this layer. However, in El Tejo there was minimal DVM of the zooplankton: instead, the increase of plankton in the epilimnion at night was due to migration from the epilimnetic sediments or from macrophytes in the littoral zone (Armengol *et al.*, 2012). The importance of the DCL for zooplankton is thus likely dependent on the degree of DVM, which can vary seasonally as the relative amounts and qualities of the seston in the epilimnion and metalimnion change (Brindza, 2002; Matthews & Mazumder, 2005). Additional work will be necessary to determine the importance of these temporal shifts, and the significance of the DCL for the upper trophic levels in a variety of lakes.

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Cyclically parthenogenetic rotifers and the theories of population and evolutionary ecology

Manuel Serra, Eduardo M. García-Roger, Raquel Ortells and María José Carmona*

Cavanilles Institute of Biodiversity and Evolutionary Biology, University of Valencia (Spain).

* Corresponding author: carmona@uv.es

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ABSTRACT

Cyclically parthenogenetic rotifers and the theories of population and evolutionary ecology

Cyclically parthenogenetic rotifers are facultative sexual aquatic microinvertebrates that live in continental and coastal waters and attracted the scientific interest of Professor M. R. Miracle. Some of her early studies anticipated the use of these rotifers as model organisms to test hypotheses in population and evolutionary ecology. This short review is aimed to stress the research she initiated at the University of Valencia. With this aim in mind, we enumerate and comment on (1) the biological and ecological features of rotifers that make them remarkable model organisms and (2) the research fields influenced by rotifer population biology. Among the latter, we selected some topics in order to illustrate how rotifer studies have contributed to our knowledge: phenotypic plasticity, competition and coexistence among cryptic species, the evolution of sex and complex life cycles, and aging. We deeply review studies on rotifer ecological genetics and evolutionary ecology with an emphasis on population studies conducted in eastern Spain and fostered by Professor Miracle. We conclude that rotifer populations harbor high local genetic diversity, with the occurrence of clonal selection during the planktonic growing season. Moreover, studies show that they have high population differentiation, which holds signatures of historical events (e.g., glacial refugia and posterior range expansion). Additionally, differential local adaptation occurs even among neighboring populations. Two remarkable conclusions are that (1) population differentiation in rotifers is most likely due to persistent founder effects rather than to "isolation by adaptation" and (2) rotifer populations can differentially adapt to the levels of environmental uncertainty in their respective localities. This occurs by adjusting the timing of sex and initiating sex and diapausing egg production earlier when populations inhabit localities with higher uncertainty. Related to environmental uncertainty but also to other environmental features (novel environments where recombination is needed to fuel natural selection, nutrient availability, etc.), experimental evolution studies have found that laboratory populations evolve quickly, allowing them to become easily adapted to new conditions. We suggest that rotifers should be used in the close future to address additional central hypotheses in evolutionary ecology as a result of the feasibility of experimental evolution and resurrection ecology on one hand and "omics" tools on the other hand. As specific topics for future research, we highlight the evolution of sex, speciation, eco-evolutionary dynamics and the regulation of complex life cycles in relation to environmental cues.

Key words: cyclical parthenogenesis, zooplankton, population differentiation, local adaptation, cryptic species, competitor coexistence, sexual reproduction, M.R. Miracle

RESUMEN

Los rotíferos partenogenéticos cíclicos y las teorías de ecología de poblaciones y evolutiva

Los rotíferos partenogenéticos cíclicos son microinvertebrados acuáticos sexuales facultativos de aguas continentales y costeras, que despertaron el interés científico de la profesora M. R. Miracle. Algunos de sus primeros estudios anticiparon el uso de estos rotíferos como organismos modelo para comprobar hipótesis en ecología de poblaciones y evolutiva. En esta breve revisión pretendemos destacar las investigaciones que inició en la Universitat de València. Así, enumeramos y comentamos (1) las características biológicas y ecológicas de los rotíferos que los convierten en organismos modelo, y (2) los campos de investigación impulsados por la biología de poblaciones de rotíferos. Entre estos últimos, hemos seleccionado la plasticidad fenotípica, las especies crípticas y la coexistencia de competidores, la evolución del sexo y los ciclos de vida complejos, y el *envejecimiento, para mostrar cómo los estudios de rotíferos han contribuido al conocimiento. A continuación, revisamos más detalladamente los estudios sobre genética ecológica y ecología evolutiva en rotíferos, destacando las contribuciones en estudios realizados en España oriental, los cuales fueron incentivados por la profesora Miracle. Concluimos que en las poblaciones de rotíferos existe una gran diversidad genética local, y se produce selección clonal durante el periodo de crecimiento planctónico. Además, los estudios muestran la alta diferenciación interpoblacional, que mantiene la huella de eventos históricos (por ejemplo, la existencia de refugios glaciales y de expansiones de rango posteriores). Además, la adaptación local diferencial ocurre incluso entre poblaciones vecinas. Dos conclusiones notables son: (1) la diferenciación poblacional en rotíferos se debe probablemente a los efectos persistentes del fundador, más que al "aislamiento por adaptación", y (2) las poblaciones de rotíferos pueden adaptarse diferencialmente a los niveles de incertidumbre ambiental de sus localidades. Esto último ocurre mediante un ajuste del momento de la reproducción sexual, iniciando la producción de huevos de diapausa de forma más temprana en las poblaciones de localidades con mayor incertidumbre ambiental. En relación con la incertidumbre ambiental, pero también con otras características ambientales (ambientes nuevos donde la recombinación es necesaria para que se produzca la selección natural, disponibilidad de nutrientes...), los estudios de evolución experimental han encontrado que las poblaciones de laboratorio evolucionan rápidamente para adaptarse a las nuevas condiciones. Como prospectiva, sugerimos que, en un futuro cercano, con los rotíferos se abordarán hipótesis adicionales de ecología evolutiva, por un lado, como resultado de la viabilidad de la evolución experimental y de la ecología de la resurrección y, por el otro, por el desarrollo de las herramientas "ómicas". Para terminar, proponemos temas específicos para futuras investigaciones: evolución del sexo, especiación, dinámicas eco-evolutivas, y regulación de ciclos vitales complejos en relación con señales ambientales.*

Palabras clave: *partenogénesis cíclica, zooplancton, diferenciación poblacional, adaptación local, especies crípticas, coexistencia entre competidores, reproducción sexual, M.R. Miracle*

Preface

Among the numerous papers authored by Professor María Rosa Miracle, two of them —published in the first period of her scientific life— can now be regarded as anticipatory clues for the role that rotifers would play in developing and testing theories in evolutionary and population ecology. In the first, Miracle (1974) used rotifer population densities recorded after a sampling campaign in Banyoles Lake for her Ph. D. thesis. Using this database, she applied a principal component analysis in an approach in which one can feel the signature of her major professor, Ramón Margalef. As a result, Prof. Miracle was able to identify niche partitioning in an assemblage of rather similar species (congeneric rotifers) dwelling in a rather spatially homogeneous environment (the planktonic environment). Not surprisingly, this work attracted the attention of George E. Hutchinson, the great limnologist who chaired the American Society of Naturalists (i.e., an association devoted to the study of evolution; Hutchinson, 1959). Regarding Prof. Miracle's approach, Hutchinson (1979) wrote, "This mode of proceeding is perhaps the purest type of niche analysis available". The second of the papers was coauthored by Charles E. King (King & Miracle, 1980) after a short stay by Prof. Miracle at Oregon State University and was first presented at the second International Rotifer Symposium. Miracle contributed to establishing these symposia by following the initiative of Agnes Ruttner-Kolisko from the very beginning, thus fueling rotifer research and networking around it. King & Miracle (1980) helped to create a paradigm in rotifer research. Accordingly, genetic population analysis, frequently using molecular markers, was applied to populations and combined with life-table experiments in order to obtain insight into the ecological interpretation of naturally occurring genetic variation. This paradigm is still at work. Among others, these two papers contributed to establishing rotifers as model organisms in hypothesis-driven research in both population and evolutionary ecology. In relation to the latter scientific field, these papers helped to show that small aquatic animals could be used in a field traditionally dominated by the study of large, terrestrial organisms (Rodríguez, 2016). Not less important, Prof. Miracle brought these seminal ideas to the University of Valencia (Spain) and fostered a group of students strongly committed to developing them. Prof. Miracle combined this approach with extensive field studies. As a member of a limnology team led by R. Margalef during 1980-81, she participated in a sampling campaign of coastal ponds and lagoons. The resulting data would provide a crucial base for the study of rotifer populations in eastern Spain. Limnology practiced by the school of ecologists founded by R. Margalef gives the most importance to physical-chemical factors. Not surprisingly, Prof. Miracle realized the role of salinity and temperature in rotifer species distributions (Miracle *et al.*, 1987) and life history traits (Miracle & Serra, 1989). These results are still inspiring current research in studies of the *Brachionus plicatilis* species complex (Montero-Pau & Serra, 2011; Gabaldón *et al.*, 2013, 2015, 2017).

INTRODUCTION

Rotifers (i.e., wheel bearers) are microscopic, aquatic invertebrates that mostly inhabit lakes, ponds, streams and coastal marine habitats. More than 2000 species have been named in the phylum Rotifera, and these have been grouped into three major clades, which are regarded as classes among many taxonomists (Bdelloidea, Monogononta, and Seisonidea). Seisonids (only four species) are obligatory sexuals; bdelloids (> 360 taxonomic species) are animals with a worm-like body and obligatory asexuality; monogononts (> 1600 named species) are facultative sexuals. It has been proposed that rotifers cannot be a monophyletic clade and that Bdelloidea and Monogononta are closer to Acanthocephala than to Seisonidea (Mark Welch, 2000; Sielaff *et al.*, 2016). Fontaneto & De Smet (2015) and Wallace *et al.* (2015) provide excellent updated information on the biology and general ecology of rotifers.

Population ecology and evolutionary ecology are two closely related fields, and they have been strongly linked with population and quantitative genetics since their very early development, when a trend to unify these fields into a single research programme (*sensu* Lakatos, 1970) was a common theme (McIntosh, 1985). The development of these fields has been driven by theory, i.e., models (e.g., the logistic model), principles (e.g., competitive exclusion), concepts (e.g., the niche concept), and laws or rules (e.g., Bergman's rule). Concomitantly, this approach uses analysis based on the "isolation of problems" (methodological reductionism) as well as simplifying assumptions, which has been problematic to naturalists and ecologists who address the complexity of natural phenomena. To some extent, this criticism misses the important point of the role of simplification in theoretical development. For instance, no biologist expects the exponential growth model to describe the dynamics of a population over an extended period, just as no physicist expects the real movement of an object to be described only by the inertia principle (see, Turchin, 2001, for an elaboration of this analogy), which does not diminish the role of simple models in organizing scientific thought and promoting progress (e.g., the logistic model allowed the development of the *r-K* strategies scheme). Nevertheless, criticism stands. A long time ago, Park (1946) stated that "modern" studies on population ecology include natural populations, laboratory populations and "theoretical populations". Regardless of this assertion, important empirical gaps still exist. Good-quality, descriptive empirical studies on natural populations are abundant and have inspired theoretical ecologists. In contrast, empirical tests of explanatory hypotheses derived from theory have been much delayed. Two obvious factors contributing to this delay are the cost and practical constraints involved in laboratory and field studies, in which confounding factors must be controlled in order to test specific hypotheses. These shortcomings may be partially overcome by using model organisms. Model organisms focus research efforts and thus allow information on their biology to be accumulated. As a result, important synergisms in our knowledge arise. Obviously, there is a trade-off here, as a handful of model organisms are not sufficient to account for the diversity of life. We need a number of cases that range in body size, typical population size, organizational complexity, trophic level, life cycle, etc.

In this short review, we aim to show the realization and the potential of cyclically parthenogenetic rotifers (i.e., rotifers in which sexual and asexual reproduction are facultative) as model organisms to improve our understanding of population dynamics, population structure, and some crucial evolutionary processes, namely, population differentiation (including phylogeography), adaptation and speciation. With this aim in mind, admittedly, the present review is not exhaustive but will stress points that have not been stressed in other recently published reviews on rotifers as model organisms in population and evolutionary studies (e.g., Fussmann, 2011; Snell, 2014; Declerck & Papakostas, 2017; Stelzer, 2017). We (1) focus on the general topics in which rotifer research has made a significant contribution and show the methodological advantages of the use of rotifers, particularly if the effort is concentrated on a few species and ecosystems. To a large extent, (2) this review is mainly based on studies in which we —the authors— were involved. This is our way of showing the effects of the approach that Prof. Miracle brought to the University of Valencia. Additionally, (3) we will highlight a perspective on the studies on cyclically parthenogenetic rotifers as a continuation of the observed tendencies.

CYCLICALLY PARTHENOGENETIC ROTIFERS: FEATURES AND ASSOCIATED METHODOLOGICAL ADVANTAGES

Rotifers are among the smallest and most short-lived and quickly reproducing metazoans. Their body size ranges from 40 to 3000 µm, although most rotifers measure from 100 to 500 µm (Hickman *et al.*, 1997). This microscopic size permits the maintenance of large laboratory populations in small volumes, while the size is large enough to allow the easy observation, manipulation and measurement of individuals (Table 1). As stated by Miracle & Serra in their review in 1989, the lifespan of cyclically parthenogenetic rotifers is typically 3-20 days (see also Nogrady *et al.*, 1993), and the lifetime reproductive output of asexual females can reach approximately 20 daughters (King & Miracle, 1980; Halbach, 1970; Walz, 1987; Carmona & Serra, 1991; Gabaldón & Carmona, 2015). Unlike other zooplankters that produce clutches of more than one offspring (e.g., cladocerans and copepods), these rotifers produce offspring sequentially (birth-flow populations; Stelzer, 2005). This has been interpreted as a constraint imposed by the large offspring size relative to the female body mass (14-70 %; e.g., Walz, 1983; Stelzer, 2011a). However, rotifers have the highest intrinsic rates of population growth among multicellular animals (Bennett & Boraas, 1989), mostly due to their short generation times. For instance, *Brachionus plicatilis* matures at the age of 24 hours (Temprano *et al.*, 1994) at 25 °C and 12 g/L salinity and has generation times of approximately 3 days. This results in an intrinsic rate of population growth as high as 0.6 days^{-1} (Miracle & Serra, 1989; Carmona & Serra, 1991), which is equivalent to doubling the population density every 1.2 days. Their rapid growth and short generation times make rotifers ideal organisms to study rapid trait evolutionary responses (Fussmann, 2011; Declerck & Papakostas, 2017; Tarazona *et al.*, 2017) and to obtain comprehensive time series of data over many generations within a short experimental time (e.g., Serra *et al.*, 2001).

Most cyclically parthenogenetic rotifers are planktonic filter feeders and may be described as euryphagous, typically feeding on bacteria, algae, protozoa, and yeast, as well as organic detritus (Wallace *et al.*, 2015). Although the species found in different environments often differ in their tolerance to ecological factors, their opportunism and wide ecological adaptability allow a number of species to be easily cultured and maintained —using simple and inexpensive diets— in controlled laboratory environments, including automated intensive continuous-culture systems (chemostats; Walz, 1993). So far, these rotifers are the only aquatic metazoans that have been found to be able to grow under steady-state conditions in semi-continuous and continuous cultures. As a result, they have become proven models for investigating population dynamics (e.g., Booras & Bennett, 1988; Rothhaupt, 1990; Ciros-Pérez *et al.*, 2001; Fussmann *et al.*, 2003; Gabaldón *et al.*, 2015) and addressing experimental evolution (e.g., Fussmann, 2011; Declerck *et al.*, 2015; Declerck & Papakostas, 2017; Tarazona *et al.*, 2017). It is worth noting that a substantial portion of the physiological and demographic information allowing the recognition of this status of rotifers came from applied studies. It is a consequence of using rotifers in aquaculture as living

Table 1. Features of cyclically parthenogenetic rotifers that make them suitable model organisms for studies on population and evolutionary ecology in metazoans. *Características de los rotíferos partenogenéticos cíclicos que los convierten en organismos modelo adecuados para estudios de ecología poblacional y evolutiva en metazoos.*

Trait (typical or general)	Methodological advantage	Field of study (examples)
Small size	Maintenance of large populations in small volumes under lab conditions.	Laboratory studies on population and evolutionary ecology.
Sufficiently large size	Ease of handling, allowing individual measurement and manipulation.	Laboratory studies; Biometrics; Demography.
High growth rate and short generation time	(1) Rapid evolutionary responses; (2) Ease of obtaining data over many generations; (3) Reasonable experimental times.	Micro-evolutionary studies; Experimental evolution; Local adaptation; Eco-evolutionary dynamics.
Generalist filter feeders	Maintenance in simple and inexpensive culture media.	Laboratory studies on population and evolutionary ecology.
Ecological adaptability	Ease of culturing and maintenance in controlled lab environments.	Laboratory studies on population and evolutionary ecology.
Eutely	Tissue cells are not renewed after ontogeny.	Physiological development and the evolution of aging.
Complex life cycle	Asexual and sexual reproduction in the same genetic background.	Ecology and evolution of sex; Life-history evolution; Trade-offs.

Cont.

Table 1. (cont.)

Clonal proliferation	(1) Breeding large cohorts of genetically identical animals; (2) Maintenance of individual genotypes for many generations in the lab; (3) Replication of genotypes in experiments; (4) Control of genetic variation in experiments.	Gene–environment interactions; Heritability; Phenotypic plasticity; Epigenetics.
Environmental sex induction	Control of sexual reproduction under experimental conditions.	Ecology and evolution of sex.
Haploid males	Development of inbred lines in the laboratory.	Population inbreeding and outbreeding.
Production of diapausing eggs	(1) Long-term maintenance of stocks; (2) Assessment of natural population genetic diversity through the sampling of diapausing egg banks; (3) Ease of clonal lineage foundation; (4) Reconstruction of evolutionary trajectories of natural populations through time via the "resurrection" of genotypes.	Laboratory studies; Ecological and genetic studies; "Resurrection ecology"; Dynamics and evolution of diapause and related bet-hedging strategies.

food for fish and crustacean larvae (Lubzens *et al.*, 1989, 2001; Hawigara *et al.*, 2007; Kostopoulou *et al.*, 2012) and in ecotoxicological tests (e.g., Snell & Carmona, 1995; Snell & Joaquim-Justo, 2007; Dahms *et al.*, 2011).

Rotifer development is direct —without a larval stage— and eutelic (no cell division occurs in the postembryonic period). Rotifers consist of approximately 1000 somatic nuclei, and their oocyte number is fixed at birth (e.g., Gilbert, 1983; Clement & Wurdak, 1991). Despite being composed of only a few cells, rotifers present remarkable anatomic complexity and have specialized organ systems, including digestive, reproductive, nervous, and osmoregulatory systems. Their eutely —in addition to their short lifespan, rapid growth and ease of culturing— makes them excellent research animals for studies on aging because the tissue cells are not renewed, allowing the investigation of specific theories of senescence (e.g., Carmona *et al.*, 1989; Enesco, 1993; McDonald, 2013; Snell, 2014).

Several of the characteristics that make cyclically parthenogenetic rotifers valuable in population and evolutionary ecological studies pertain to their complex life cycle (Box 1, Fig. 1), which includes multiple generations (Moran, 1994). They are capable of both clonal proliferation through parthenogenesis and sexual reproduction. Clonal reproduction is a unique and powerful experimental tool because high numbers of isogenic individuals (naturally produced clonal lines) can be obtained and maintained for prolonged periods. This allows for replication and comparisons of (1) various environments against a defined genetic background or (2) various genotypes against a defined environment.

BOX 1. Cyclical parthenogenesis in rotifers.

Populations of cyclically parthenogenetic rotifers are typically temporal in the plankton and recolonize the water column during the planktonic growth cycle through the hatching of diapausing eggs from pond and lake sediments (Fig. 1). The hatchlings are asexual (i.e., amictic) females that produce diploid, subitaneous eggs that —barring mutations— develop into genetically identically asexual females (clonal proliferation). The sexual phase begins with the parthenogenetic production of sexual (i.e., mictic) daughters by asexual mothers as a fraction of their offspring in response to environmental inducing factors. Sexual females produce meiotic haploid eggs that develop parthenogenetically into haploid males, and if young sexual females mate, their haploid eggs are fertilized and develop into diapausing eggs (actually cysts). These eggs can survive adverse conditions and enable populations to recolonize the water column when suitable conditions resume at the site and also disperse to other habitats. After receiving appropriate stimuli, a fraction of the diapausing egg hatch into asexual females, and a new growing season begins. The unhatched fraction of diapausing eggs accumulate in the sediment, forming banks.

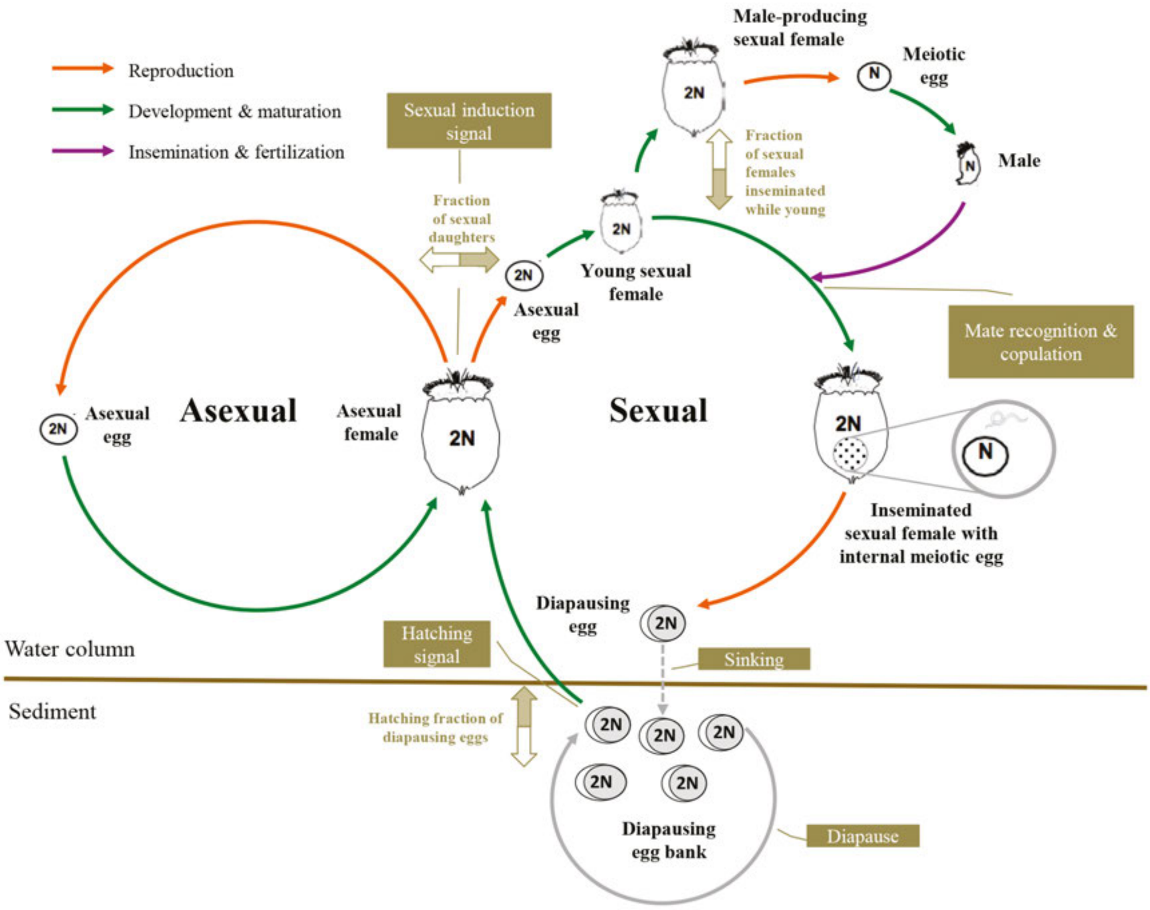

Figure 1. Life cycle of monogonont rotifers (modified from Serra *et al.*, 2018). *Ciclo vital de rotíferos monogonontes (modificado a partir de Serra* et al.*, 2018).*

This facilitates genetic and environmental influences on the phenotype to be conveniently separated in experimental settings, which allows evolutionary ecology questions that are otherwise difficult to approach (e.g., phenotypic plasticity, the genomic basis of ecologically relevant traits, changes in gene expression in response to environmental conditions, and epigenetic phenomena) to be addressed.

In cyclically parthenogenetic rotifers, sexual reproduction is dependent on environmental factors that may differ among genera or species, such as the photoperiod, population density, and diet (e.g., Gilbert, 1974; Pourriot & Snell, 1983; Schröder, 2005). Therefore, for instance, the population density —which acts as an inducing cue in the genus *Brachionus*— can be used in the laboratory to experimentally manipulate sex initiation, as studied by Prof. Miracle and coworkers (Carmona *et al.*, 1993, 1994; see also Stelzer & Snell, 2003). This is useful in studies examining relevant aspects of the ecology of sexual reproduction (see next section). During sexual reproduction, asexual females produce parthenogenetically sexual females as some fraction of their offspring. That is, asexual reproduction does not stop, and the two reproductive modes co-occur in the population. Thus, the level of sexual reproduction (i.e., the fraction of sexual females) can be correlated with environmental factors and habitat characteristics to analyze the optimization of investment into sexual reproduction (Serra *et al.*, 2004). While in cladocerans —the other group of cyclical parthenogenetic zooplankters— the same female can produce meiotic and ameiotic eggs, in rotifers, these two types of eggs are produced by different females. Only the oocytes of so-called sexual (or mictic) females undergo meiosis, and they develop into haploid males (if not fertilized) or diploid diapausing eggs (if fertilized). Therefore, the sex-determination system in rotifers is haplodiploid, and because each male represents a random haploid sample of its mother genome, mating between males and sexual females of the same clone is genetically equivalent to selfing. This allows for the easy development of inbred lines and the study of inbreeding depression effects (Birky, 1967; Tortajada *et al.*, 2009), although controlled reproductive crosses are very laborious to undertake. Another feature of cyclically parthenogenetic rotifers that makes them useful for examining the evolutionary maintenance of sex (e.g., investment into sexual reproduction and the cost of sex) is that sexual and asexual females are virtually identical in morphology and, if belonging to the same clone, have the same genetic background. This facilitates the comparison of the life-history traits of females differing only in their reproductive mode (e.g., Carmona & Serra, 1991; Gilbert, 2003; Snell, 2014; Gabaldón & Carmona, 2015) or in the proportion of sexual daughters produced (e.g., Carmona *et al.*, 1994; Fussmann *et al.*, 2007) without the interference of other phenotypic variation (King, 1970). Given the morphological similarity between asexual and sexual females, they have to be identified based on their eggs. Thus, a caveat is that neonate and non-ovigerous females cannot be classified, resulting in a smaller practical sample size for the calculation of the level of sexual reproduction.

An additional feature distinctive of cyclically parthenogenetic rotifers associated with their life cycle is that the development of sexually produced eggs is halted temporarily during a resting stage —i.e., sex and diapause are linked (Schröder, 2005). The arrested embryos can survive adverse conditions and remain viable for decades, providing dispersal in both space and time (Kotani *et al.*, 2001; García-Roger *et al.*, 2006a). Not all diapausing eggs hatch when favorable conditions occur; instead, some of them remain viable in the sediment for longer periods, forming egg banks (Evans & Dennehy, 2005). In terms of methodological advantages, diapausing rotifer eggs provide (1) the long-term maintenance of culture stocks, (2) the rapid and cost-effective assessment of the genetic diversity of natural populations through the sampling of diapausing egg banks instead of sampling rotifers from the water column, (3) the easy establishment of clonal lines in the laboratory, and (4) the investigation of past rotifer populations in the field. Regarding the last point (i.e., resurrection ecology; Brendonck & De Meester, 2003), the possibility of measuring evolutionary change by comparing past populations to current ones is made feasible by sampling diapausing egg banks in lake or pond sediments, which also include a record of environmental changes (Hairston *et al.*, 1999; Piscia *et al.*, 2016; Zweerus *et al.*, 2017).

Working with rotifers poses challenges in addition to those already mentioned. First, rotifer cultures are not free from crashes and contamination (e.g., by ciliates). These are problems that are not exclusive to rotifers but shared with all other experimental organisms. Luckily, the opportunity to use continuous-culture techniques (e.g., chemostats) for rotifers is helping cultures to be maintained for extended periods without contamination (see Declerck & Papakostas, 2017). In addition to that challenge, it is also worth mentioning that complete genome data for monogonont rotifers are still very limited, with the only exception of *Brachionus calyciflorus* and *B. plicatilis*, for which genome assembly information is recently available (Kim *et al.*, 2018; Franch-Gras *et al.*, 2018).. However, genomic tools are increasingly affordable for research groups, and other partial-genome approaches have been successfully implemented in rotifers (e.g., Mark Welch & Mark Welch, 2005; Denekamp *et al.*, 2009; Montero-Pau & Gómez, 2011; Hanson *et al.*, 2013a,b; Ziv *et al.*, 2017).

TESTING HYPOTHESES REGARDING POPULATION AND EVOLUTIONARY ECOLOGY USING ROTIFERS

The attention to rotifers in ecological and evolutionary studies can be quantitatively illustrated using the number of papers published as a metric. After a search in the Thomson ISI Web of Science for “(ecol* AND evol*) AND (rotifer*)” in the topic search query, we selected papers in the field of evolutionary biology and summed the number of papers in this field from our own archives. This search yielded 706 records for the period 1966–2017. Notably, the counts per year showed an increasing trend, as also occurs for all studies in evolutionary ecology (“ecol*” AND “evol*”; Fig. 2). The topics in which rotifer research has made a significant contribution are summarized in Table 2, with references to the most representative studies. Below, we go over the main findings derived from these studies.

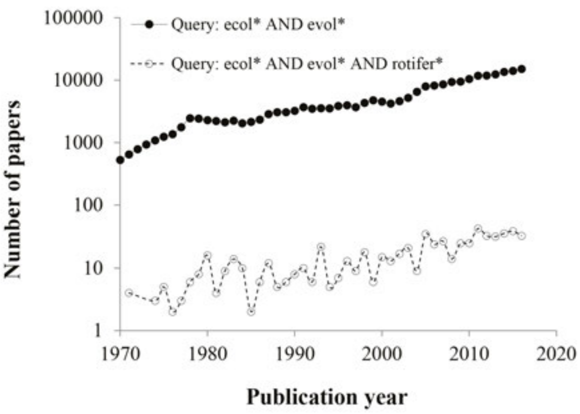

Figure 2. Absolute number of papers on ecology and evolution published every year since 1970. Depicted are the numbers of papers returned after topic search queries for all taxa (ecol* AND evol*; filled dots) and rotifers only (ecol* AND evol* AND rotifer*; open dots). Counts were obtained from the Thomson ISI Web of Science® after filtering by research domain = science and technology and database = Web of Science Core Collection. Two publications for all taxa published prior to 1970 were omitted. *Número absoluto de artículos sobre ecología y evolución publicados anualmente desde 1970. Se muestran los números de artículos resultantes de las consultas de búsqueda por tema para cualquier taxa (ecol * AND evol *; puntos negros) y para rotíferos solamente (ecol * AND evol * AND rotifer *; puntos blancos). Los recuentos se obtuvieron de Thomson ISI Web of Science® después de filtrar por Research domain = Science and technology, y Database = Web of Science Core Collection. Se omitieron dos publicaciones anteriores a 1970 para la búsqueda correspondiente a todos los taxones.*

Phenotypic plasticity

Clonally reproducing organisms, by allowing the control of genetic variation, offer an opportunity to study phenotypic plasticity (i.e., the ability of individual genotypes to produce different phenotypes when exposed to different environmental conditions; see Pigliucci *et al.*, 2006; Fusco & Minelli, 2010) and to estimate reaction norms. The thermal environment is regarded as crucial in shaping the adaptations and distributions of living beings. Not surprisingly, the developmental morphological response to temperature has been a widely studied form of phenotypic plasticity in rotifers. In many rotifer species, a larger body size is observed at low temperatures, a phenomenon also observed in other ectotherms and known as the temperature-size rule (TSR, Atkinson, 1994). In rotifers, the pioneering work of Prof.

Miracle provided support for the TSR in *B. plicatilis* (Serra & Miracle, 1983; see also Snell & Carrillo, 1984; Walczynska *et al.*, 2017) and more recently in *Synchaeta* (Stelzer, 2002) and *B. calyciflorus* (Sun & Niu, 2012). There is also important phenotypic plasticity in rotifer egg size, which was first noticed by Prof. Miracle and coworkers (Serrano *et al.*, 1989; see also Galindo *et al.*, 1993; Stelzer, 2005; Sun & Niu, 2012).

Inducible defenses —another type of phenotypic plasticity— are hypothesized to evolve when defenses are costly and predation pressure fluctuates. They have been reported to occur in rotifers, in which their occurrence is triggered by the presence of some reliable cues released by predators (Gilbert, 2009; 2011). As a consequence of the development of inducible defenses, rotifers are expected to experience fitness costs (Gilbert, 2013), although such costs can be manifested in different forms (e.g., decreased reproduction, as observed in *B. angularis*, or reduced sexual investment, as observed in *B. calyciflorus*; Yin *et al.*, 2016). Interestingly, selection exists during a season for much of this response when predators are present (Halbach & Jacobs, 1971; reviewed in Gilbert, 2018) such that developmental and selective environments overlap in their time scales. This shows that evolutionary responses may exist in rotifer populations at a typical ecological scale of observation. Using rotifers, it has been shown that inducible prey defenses enhance plankton community stability and persistence, likely through negative feedback loops that prevent strong population oscillations

Table 2. Examples of studies using rotifers in the development of population and evolutionary ecology hypotheses and theories. *Ejemplos de estudios que utilizan rotíferos en el desarrollo de hipótesis y teorías sobre ecología de poblaciones y evolutiva.*

Topic	Examples
Adaptive phenotypic plasticity	Serra & Miracle, 1983; Snell & Carrillo, 1984; Stelzer, 2005; Walczynska *et al.*, 2017.
Population differentiation and local adaptation	Campillo *et al.*, 2009; Alcántara-Rodríguez *et al.*, 2012; Franch-Gras *et al.*, 2017.
Niche differentiation	Miracle, 1974; Ciros *et al.*, 2004; Gabaldón *et al.*, 2013, Lapesa *et al.*, 2002; 2004.
Cryptic speciation	Gómez & Serra, 1995; Gómez *et al.*, 2002; Campillo *et al.*, 2005; Walsh *et al.*, 2009; Obertegger *et al.*, 2012; Mills *et al.* 2017.
Evolutionary ecology of sex	Aparici *et al.*, 1998, 2002; Carmona *et al.* 2009; Becks & Agrawal, 2010; 2012; Stelzer & Lehtonen, 2016.
Evolution of diapause	Denekamp *et al.*, 2009, 2011; Martínez-Ruiz & García-Roger, 2015; Stelzer, 2017.
Aging	King & Miracle, 1980; Carmona *et al.*, 1989. Carmona & Serra, 1991; Snell *et al.*, 2012; Snell, 2014; Snell *et al.*, 2015; Gribble & Mark Welch, 2017.
Experimental evolution	Fussmann *et al.*, 2003; Smith & Snell, 2012; Declerck *et al.*, 2015; Tarazona *et al.*, 2017.

(Van der Stap *et al.*, 2007; Aránguiz-Acuña *et al.*, 2010). These results provide support for the idea that evolutionary changes in these organisms may have consequences for the functioning of entire ecosystems (Matthews *et al.*, 2014).

Although morphology is the most studied feature, phenotypic plasticity also refers to changes in an organism's behavior and/or physiology (for a review, see Gilbert, 2017). A striking example in rotifers is the transition from the production of exclusively asexual daughters to the production of sexual and asexual daughters (see above). Because phenotypic plasticity is the result of shifts in gene expression, one powerful way to examine how rotifer genotypes respond to particular environments is to use transcriptomics, which is currently easily applicable to many ecological model systems, with rotifers not being an exception (Denekamp *et al.*, 2009; 2011; Hanson *et al.*, 2013a).

Because rotifers can show (1) remarkable phenotypic plasticity, (2) within-species genetic variation —which may involve ecologically relevant traits (e.g., Campillo *et al.*, 2009; Franch-Grass *et al.*, 2017a, see below)— and (3) cryptic speciation resulting in complexes of reproductively isolated groups with very similar morphology (see below), special care is needed in order to reliably dissect these levels of variation. Otherwise, the inaccurate identification of these phenomena may misguide the evolutionary and ecological explanations that are hypothesized. Interestingly, the association between small rotifer size and high temperature can be discomposed into differential species adaptation, within-species evolution, and co-gradient variation due to phenotypic plasticity (Walczynska & Serra, 2014a,b; Walczynska *et al.*, 2017).

Aging, at the crossroads between physiology and evolution

Complex physiological changes are involved in aging, but from a life history perspective, the result is a decrease in fitness components (i.e., survival and fecundity) with age after maturity. This poses the question of why natural selection does not act to prevent aging but most likely has selected for it. The evolutionary theory of aging is based on the notion that the strength of natural selection declines with progressive age (Rose, 1991), being widely acknowledged that high performance at a young age occurs at the cost of poor performance at an older age. Rotifers have been shown to be particularly useful in studies focused on the physiological side of the problem (for recent reviews, see Snell, 2014; Snell *et al.*, 2015). Many of the abovementioned features of monogonont rotifers, particularly eutely, their ease of culturing and their short generation times, have allowed these organisms to be considered adequate experimental organisms for the study of aging (Enesco, 1993). The most successful results of aging studies in rotifers include evidence of lifespan extension through caloric restriction (Gribble *et al.*, 2014; Snell, 2015), the supplementation of antioxidants in the diet (Snell *et al.*, 2012) or the effect of controlled environmental conditions (e.g., low temperatures; Johnston & Snell, 2016). Another advantage of rotifers in the study of aging relies on the availability of ready-for-use genomic tools that can be applied to rotifers (Gribble & Mark Welch, 2017). These new tools have allowed the discovery of genes involved in aging by comparing gene expression in individuals of different ages (Gribble & Mark Welch, 2017) as well as the identification of target genes whose expression can be altered at will by novel techniques, such as RNAi knockdown (Snell *et al.*, 2014).

Studies on the evolution of sex and life cycle traits

One of the major problems still unsolved in evolutionary biology is determining which evolutionary forces maintain sex in populations, that is, which advantages compensate for the costs of sex (Williams, 1975; Maynard Smith, 1978; Bell, 1982). Sex has inherent costs (for a review, see Stelzer, 2015) and potential advantages due to recombination (e.g., Hurst & Peck, 1996; Roze, 2012). A recurrent problem when relating sexual reproduction to environmental or genetic factors is that, for many organisms, sex follows an all-or-nothing rule. Fortunately, cyclical parthenogens have the advantage of displaying a range of investment in sexual vs. parthenogenetic reproduction (Stelzer & Lehtonen, 2016). Several studies have shown strong selection against sexual investment during the course of a growing season in *Brachionus* species or in laboratory cultures (Fussmann *et al.*, 2003; Carmona *et al.*, 2009). The direct comparison between obligate asexual and facultative sexual strains of *B. calyciflorus* has shown how the former typically outcompetes the latter (Stelzer, 2011) over the short term. Overall, these studies provide evidence for the costs of sex. Interestingly, recent experiments have shown how environmental heterogeneity could favor sexual reproduction in rotifers (Becks & Agrawal, 2010, 2012). These authors found that sex evolved at higher rates in experimental populations of *B. calyciflorus* during adaptation to novel environments in comparison to populations in which environmental conditions were kept constant and that the sexual offspring showed higher fitness variability, in agreement with the idea that sex generates new genetic combinations (Becks & Agrawal, 2012).

Another important question raised by cyclical parthenogenesis is why this cycle is not a more common cycle. Cyclical parthenogenesis is not a monophyletic trait (i.e., it has evolved several times) and has been regarded as the optimal combination of fast asexual proliferation and episodic sex. Theoretical studies predict that a little of sex is enough to fully provide the advantages of recombination while minimizing the costs (Peck & Waxman, 2000). However, this cycle is found in only approximately 15 000 animal species (Hebert, 1987) out of the estimated 7.77 million species of animals on Earth (Mora *et al.*, 2011). A sound explanatory hypothesis is that cyclical parthenogenesis is inherently unstable in evolutionary terms because its transition to obligate asexuality does not require the acquisition of a new function but only the loss of the sexual function. Moreover, when this transition occurs, the newly emerged asexual linages outcompete the cyclically parthenogenetic lineages -which have to pay the short-term costs of sex- before the long-term advantages of sex arrive. In the case of ancient cyclical parthenogens, the linkage between sex and the production of resistant stages has been suggested to be responsible for the maintenance of cyclical parthenogenesis (Simon *et al.*, 2002; Serra *et al.*, 2004). That is, recurrent adverse periods cause short-term selection for diapause, the linkage between diapause and sex causes the maintenance of sex, and this allows the long-term advantages of sex to be realized. Recent theoretical research has shown that the costs of sex decline when sex is linked to diapause (Stelzer & Lehtonen, 2017), which supports the idea that the short-term advantages of diapause counterbalance the costs of sex and prevent facultative sexuals from being displaced by obligate asexuals.

Hidden biodiversity and local species richness

A fortunate by-product of molecular marker studies when applied to what was thought to be a single species is unmasking cryptic species (also called sibling species; Gómez *et al.*, 2002a; Walsh *et al.*, 2009; Leasi *et al.*, 2013; Mills *et al.*, 2017), a phenomenon that has led to research on the development of molecular tools for species identification (Gómez *et al.*, 1998; Montero & Gómez, 2011; Obertegger *et al.*, 2012). Among metazoans, rotifers seem to have one of the highest levels of hidden diversity resulting from cryptic speciation, with at least 42 cryptic species complexes (Fontaneto *et al.*, 2009; Gabaldón *et al.*, 2017). To date, the best-studied cryptic species complex is that of *Brachionus plicatilis* (Box 2), for which a multifold approach integrating morphological and DNA taxonomy, cross-mating experiments, and ecological and physiological evaluations has been used to separate species and understand their ecological divergence and the conditions favoring their coexistence (e.g., Serra *et al.*, 1998; Ciros-Pérez *et al.*, 2001; Gómez *et al.*, 2002a; Suatoni *et al.*, 2006; Serra & Fontaneto, 2017; Mills, 2017). Because monogonont rotifers reproduce sexually during part of their life cycle (Box 1), evidence of species status can be provided through pre-mating reproductive isolation. Interestingly, contact chemoreception of a surface glycoprotein serves as a mate recognition pheromone (MRP; Snell *et al.*, 1995). Molecular and genetic studies have identified the protein and gene responsible, making rotifers a premier model for mechanistically investigating population differentiation and

BOX 2. Cryptic speciation in the monogonont rotifer species complex *Brachionus plicatilis*.

Cryptic (also called sibling) species are those having great morphological similarity such that classical, morphologically based taxonomy considers them to be a single species (Knowlton 1993; Bickford *et al.*, 2007). The understanding of the extent of cryptic diversity within any given taxon is essential not only to assess its overall diversity but also to recognize the complexity of its ecological interactions and evolutionary histories. Such knowledge is even more essential when the taxa under consideration are valuable ecological and evolutionary models, as is the case of monogonont rotifers.

The cryptic species complex *Brachionus plicatilis* is currently the best studied among rotifers. It is known to host a large amount of hidden and still not completely resolved diversity (Mills *et al.*, 2017; Serra & Fontaneto, 2017). The most recent study performed using several approaches to DNA taxonomy confirmed the existence of 15 previously described species within the complex (Mills *et al.*, 2017; Fig. 3). Several studies have demonstrated prezygotic and postzygotic reproductive isolation among some members of the complex (Ortells *et al.*, 2000; Suatoni *et al.*, 2006; Snell & Stelzer, 2005). In laboratory populations, some species have only been partially isolated. Only six species have been formally described: *B. plicatilis* s.s. (Müller, 1786), *B. rotundiformis* (Tschungunoff, 1921), *B. asplanchnoidis* (Charin, 1947), *B. ibericus* (Ciros-Pérez *et al.*, 2001), *B. manjavacas* (Fontaneto *et al.*, 2007), and *B. koreanus* (Hwang *et al.*, 2013); the remaining species have received unofficial names (Fig. 3). Morphological discrimination among some species of this complex is possible but complicated (Ciros-Pérez *et al.*, 2001; Campillo *et al.*, 2005; Fontaneto *et al.*, 2007). Despite their great morphological similarity and close phylogenetic relationships —which result in expectable similar ecological requirements (i.e., niche conservatism) and strong competitive interactions— subsets of these species often co-occur in many waterbodies of the Iberian Peninsula (Ortells *et al.*, 2003; Gómez, 2005; Lapesa *et al.*, 2004; Montero-Pau *et al.*, 2011). Information regarding the mechanisms that allow cryptic species coexistence is available for several species of the complex (*B. plicatilis*, *B. ibericus*, *B. rotundiformis* and *B. manjavacas*) based on studies of their ecological requirements, their seasonal specialization, and the characterization of their abiotic and biotic niche differentiation (Gabaldón *et al.*, 2017).

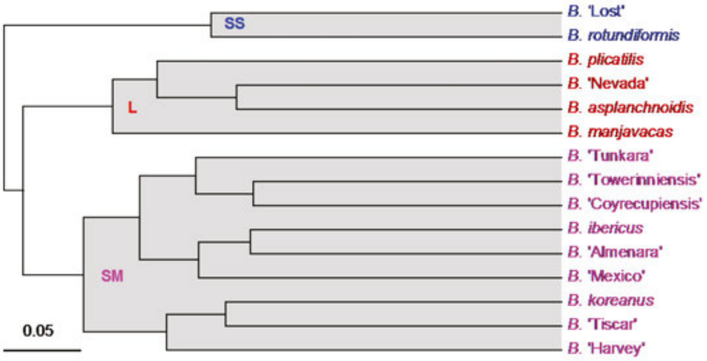

Figure 3. Phylogenetic relationships in the *B. plicatilis* species complex (modified after Serra & Fontaneto, 2017; see this publication for details) showing 15 putative species (branching within these species is not shown). Shaded clades indicate the three main groups that correspond with the classical size-based morphological classification (SS: small; SM: small-medium; L: large; see Ciros *et al.*, 2001). *Relaciones filogenéticas en el complejo de especies* B. plicatilis *(modificado a partir de Serra & Fontaneto, 2017; donde pueden verse más detalles), en las que se muestran 15 especies putativas (sin detalles de las ramificaciones dentro de especie). Los clados sombreados indican tres grupos principales correspondientes con la clasificación morfológica clásica basada en tamaño (SS: pequeño, del inglés "small"; SM: pequeño-mediano, del inglés "small-medium"; L: grande, del inglés "large"; véase Ciros* et al.*, 2001).*

cryptic speciation (Snell *et al.*, 1995, 2009; Snell & Stelzer, 2005; Gibble & Mark Welch, 2012).

Uncovering cryptic species is an important taxonomic issue in order to increase the accuracy of global biodiversity estimates. The case of the *B. plicatilis* species complex clearly shows the magnitude of the possible underestimation: what was thought to be a single rotifer species in the 1980s is currently regarded as a complex of fifteen cryptic species (Mills *et al.*, 2017). There are several important ecological implications of the uncovering of cryptic species (Gabaldón *et al.*, 2017). One is the need to re-evaluate the eurioic character and the cosmopolitan distribution of the erroneously considered single species (Gómez *et al.*, 1997). Another is the need to discriminate between within-species variation (either genetic or due to the developmental environment) and among-species variation; for instance, to know whether apparent cyclomorphosis (i.e., seasonal change in the morphology of a population) may actually be a repeated pattern of seasonal substitution of similar species (Gómez *et al.*, 1995; Ortells *et al.*, 2003). Most importantly, uncovering cryptic species allows the local species richness to be evaluated and calls for explanations for the coexistence of species that are expected to have very similar niches, resulting in strong competition. Rotifer studies have shown that the co-occurrence of cryptic species in a particular location is rather common (Ortells *et al.*, 2000; 2003; Gómez *et al.*, 2005; Lapesa *et al.*, 2004; Montero *et al.*, 2011; Leasi *et al.*, 2013). In the *B. plicatilis* species complex, seasonal oscillation in local salinity and temperature can help to explain this co-occurrence when combined with species specialization in relation to these factors (Gómez *et al.*, 1997; Montero-Pau *et al.*, 2011; Gabaldón *et al.*, 2015) so that cryptic species have seasonal differences but overlapping distributions (Gómez *et al.*, 1995; 2002a; 2007; Ortells *et al.*, 2003). However, coexistence may also be mediated by subtler niche differentiation. Thus, it has been reported that cryptic rotifer species differing in body size show (1) differential exploitative competitive ability based in resource (microalgae) use partitioning and (2) differential susceptibility to predation (Ciros-Pérez *et al.*, 2001, 2004; Lapesa *et al.*, 2002, 2004). Nevertheless, in species of the complex that are extremely similar in size, coexistence is favored by both differences in their response to fluctuating abiotic salinity and life-history traits related to diapause (Montero-Pau *et al.*, 2011; Gabaldón *et al.*, 2013, 2015; Gabaldón & Carmona, 2015). On one hand, investment in diapause by a population gives short-term advantages to its competitors; for instance, such investment by a superior competitor may provide an opportunity for coexistence to inferior ones (Montero-Pau & Serra, 2011). On the other hand, diapausing eggs Cwhich are insensitive to competition— allow for the temporal escape from competition as they wait in the sediment for a favorable time window in the water column (e.g., Gabaldón *et al.*, 2015).

POPULATION DIFFERENTATION AND LOCAL ADAPTATION IN ROTIFERS

As in many other taxa, the study of population differentiation and local adaptation in rotifers sheds light on several crucial topics in ecology and evolution. First, it provides signatures of an evolutionary past, as evidenced by phylogeography studies (i.e., the phylogenetic analysis of geographic patterns; Gómez *et al.*, 2000; 2002b; 2007; Campillo *et al.*, 2011a). Second, it identifies the impact of natural selection (1) on the formation and persistence of populations by distinguishing the effects of local adaptation from those of genetic drift (Campillo *et al.*, 2009; Franch-Grass *et al.*, 2017a) and (2) on the temporal patterns —either periodic or non-periodic— of genetic change. Third, population differentiation is the first step in what might end in speciation. Last but not least, as stated above, such studies may uncover the existence of cryptic speciation (Mills *et al.*, 2016).

Intrapopulation studies

The within-population genetic diversity in cyclically parthenogenetic rotifers, as assessed from molecular marker studies, is typically very high (Gómez & Carvalho, 2000; Ortells *et al.*, 2006; Montero-Pau *et al.*, 2017). This finding is expected due to their large effective population sizes and suggests that local populations do not suffer from bottlenecks. In fact, diapause, as a potential bottleneck, does not work in this way, likely because the abundance of diapausing eggs in sediment banks is on the order of millions even in small ponds (García-Roger *et al.*, 2006b; Montero *et al.*, 2017). Allele frequencies in the water column often show deviations from Hardy-Weinberg expectations (HWE; Gómez & Carvalho, 2000; Ortells *et al.*, 2006). This might be due to the Wahlund effect (i.e., a reduction in the overall heterozygosity of a population as a result of the subpopulation structure) if the genotypes in the water column are a result of those from diapausing eggs in the sediment bank produced both at different times and under different selection pressures. Alternatively, deviation from HWE could be the result of clonal selection during parthenogenetic proliferation. Gómez & Carvalho (2000) demonstrated clonal selection by the end of the growing season, and Ortells *et al.* (2006), by comparing different populations, found a correlation between (1) the clonal diversity harbored by a population and (2) the duration of the growing season. Both studies reported high genetic diversity at the start of the growing season, whereas allele frequencies strongly deviated from those expected from genetic equilibrium by the end of the season. These studies suggest that the hatching of diapausing eggs provides high genotypic diversity when the population is established at the start of the growing season. However, this diversity is eroded by clonal selection during parthenogenetic proliferation (i.e., the longer the growing season, the lower the genetic diversity).

Fluctuating selection seems to act in some cases and traits. For instance, Carmona *et al.* (2009) reported a decrease in the propensity for sexual reproduction over the growing season as a result of the short-term costs of sex and diapause (i.e., a decreased rate of parthenogenetic proliferation). This selection for low investment in sex should reverse between growing seasons, as diapausing eggs are essential for survival during adverse periods (see above). The occurrence of fluctuating selection with a repeated annual pattern was also suggested by Papakostas *et al.* (2013). In this study, genotypes of a single species in a single locality clustered into groups with strong genetic divergence and differential temporal distribution, suggesting differential seasonal specialization. This study opens a window to the possibility of allochronic sympatric speciation in zooplankters, a hypothesis that was formulated a long time ago (Lynch, 1984).

Interpopulation studies: population differentiation, local adaptation and phylogeographic structure

The traditional view regarding small (< 1 mm) organisms states that, due to their large dispersal capability, (1) these species do not present biogeographic restrictions and should lack geographic structure (Finlay, 2002) and (2) the populations of a species should be connected by gene flow, hindering geographic speciation. This view has been challenged by the high genetic differentiation found in many continental zooplankters after assessments using molecular markers. For instance, species of the genus *Brachionus* show strong genetic differentiation among populations, even among those living in nearby localities (Gómez *et al.*, 2002; Derry *et al.*, 2003; Campillo *et al.*, 2009; Franch-Gras *et al.*, 2017a). Gene flow seems to be so restricted that it has not blurred the signature of historical events. Consistently, phylogeographic analyses have shown that rotifer populations in the Iberian Peninsula exhibit a within-species differentiation structure that might reflect the impact of Pleistocene glaciations (Gómez *et al.*, 2000; 2002b; Campillo *et al.*, 2011a). Accordingly, this structure seems to be due to the serial recolonization of ponds from glacial refugia located in southern Spain. Historical effects are diluted only at small geographic scales, likely due to the intense dynamics of extinction and recolonization from neighboring localities that are still genetically differentiated (Montero-Pau *et al.*, 2017).

The disagreement between the traditional view and the empirical evidence stressed above has been termed the "dispersal-gene flow paradox" (i.e., high dispersal capacity contrasts with pronounced genetic differentiation among neighboring populations; De Meester *et al.*, 2002). The hypothetical explanation for this paradox is based on strong persistent founder effects due to the combination of (1) populations founded by a few individuals —with the important corresponding sample effect, (2) fast proliferation, and (3) the accumulation of large diapausing egg banks. These factors would quickly create large population sizes after the establishment of a population from a few colonizers such that later immigrants are diluted within a large population and have little effect. Under these conditions, the time necessary to reach the migration-drift equilibrium would be so long that it would not be observed due to the interference of major historical changes (e.g., speciation, climate change). Moreover, it has been postulated that local adaptation can also quickly occur, reinforcing barriers against immigration ("the monopolization hypothesis", De Meester *et al.*, 2002). Rotifers support some assumptions of these explanations. At a large geographical scale, Gómez *et al.* (2002a) found levels of population differentiation that were consistent with initial colonization by single resting eggs from neighboring populations. Additionally, the establishment of populations of *B. plicatilis* in newly created ponds in a restored marshland followed by Badosa *et al.* (2017) revealed a low number of founding clones. Nevertheless, colonization might exhibit rather complex dynamics. The effect of the very first founders can eventually decline if later immigrants have a selective advantage over the highly inbred local residents, an effect experimentally demonstrated in *B. plicatilis* by Tortajada *et al.* (2010). Therefore, the establishment of a viable population might occur during a time window scaled by a decrease in inbreeding depression due to an increase in genetic diversity. In addition, diapausing egg banks may initially be relatively small or lack ecologically relevant variation, reducing their buffering role against immigrant genes. In their study, Badosa *et al.* (2017) consistently found effective gene flow soon after foundation. In rotifers, differentiation in molecular markers and differentiation in ecologically relevant traits are poorly correlated (Campillo *et al.*, 2011b). Thus, local adaptation does occur in rotifers, but it seems to be less important than persistent founder effects in preventing effective gene flow (i.e., in causing population differentiation). This could differ from what has been observed in cladocerans, in which population sizes are typically lower than those in rotifers; cladocerans also live in relatively more constant environments, indicating that local adaptation is a factor in the observed population differentiation in that taxon (De Meester *et al.*, 2004).

Due to the effective clonal selection that occurs during the parthenogenetic phase and the decrease in genetic variation that occurs through recurrent sexual recombination, cyclical parthenogens are expected to be prone to local adaptation (Lynch & Gabriel, 1983), particularly because, as stated above, the effective gene flow is low. Research on local adaptation in rotifers has benefited from the potential to perform common garden experiments. Ideally, reciprocal transplant experiments demonstrate local adaptation by showing that the "local vs. foreign" (i.e., the average fitness of local genotypes is higher than the average fitness of foreigners) or "home vs. away" (i.e., the average fitness of a genotype is higher in its native locality than in other localities) criterion is fulfilled (see Kawecki & Ebert, 2004). However, this kind of experiment is logistically complicated, as it requires introducing genotypes from natural populations from each of ≥ 2 environments into the others. As an alternative, common garden experiments have allowed the study of the fitness response of different rotifer genotypes when cultured under laboratory conditions mimicking the typical values of very specific environmental variables in natural populations. Campillo *et al.* (2011b) measured fitness components (e.g., the intrinsic rate of increase) in the laboratory under combined salinity and temperature conditions in *B. plicatilis* populations sampled from six localities. The variation found therein was associated with the actual conditions of the ponds from which they were sampled, and a clear case of local adaptation to high salinity was reported (Campillo *et al.*, 2011b). This adaptation to local salinity is consistent with the fact that species specialization exists in relation to this parameter in rotifers inhabiting brackish waters (Miracle & Serra, 1989). Campillo *et al.* (2011) also found signatures of life cycle adaptations to habitat uncertainty. A long time ago, rotifer populations in unpredictable habitats were proposed to invest early and continuously in sexual reproduction during their annual growth cycle (a bet-hedging strategy; Carmona *et al.*, 1995; Serra & King, 1999; Serra *et al.*, 2004, 2005), but variation in traits could not be correlated with an estimate of unpredictability. Recently, Franch-Gras *et al.* (2017b) used time series obtained from remote sensing data to estimate the degree of unpredictability in inland ponds of eastern Spain, as indicated by the long-term fluctuations in the water surface area of the ponds. After the observation of a rather wide range in unpredictability, they studied life-history traits associated with diapause (Franch-Gras *et al.*, 2017a). One of the hypotheses addressed was a higher propensity for sex with increasing unpredictability, since early sex means early investment in diapausing eggs —at the cost of decreasing the rate of clonal proliferation—, and investing early in diapause is needed to prevent growing seasons from being unexpectedly short. Their results showed the expected positive correlation between habitat unpredictability and the propensity for sex, this being one of the few studies testing bet-hedging strategies allowing adaptation to unpredictable environmental fluctuations. This adaptation is possible because, as observed in a recent study using experimental evolution, rotifers quickly evolve bet-hedging strategies in response to environmental unpredictability (Tarazona *et al.*, 2017).

Recently, Declerck *et al.* (2015) took a further step in the study of adaptation to the local environment by means of what was called a common garden transplant approach. In their study, naturally derived populations of *B. calyciflorus* were first subjected to two contrasting selective regimes related to P enrichment (P poor vs. P rich) in chemostats. Later, rotifers with different genotypes from each selective regime were grown under both P-poor and P-rich conditions, and population performance estimates (growth, yield, grazing pressure) were used to demonstrate rapid adaptation (within a growing season) in the populations. This observation is somewhat consistent with the "local vs. foreign" criterion mentioned above.

PROSPECTS

In this review, we have shown how cyclically parthenogenetic rotifers are remarkable because of the features of their reproductive biology, which have enabled (1) exceptional experimental flexibility and control, (2) the collection of an extensive amount of both ecological and life-history trait data for many rotifer species, and (3) their use in tests of specific hypotheses in population and evolutionary ecology studies. Several of these studies open the door to a series of questions concerning their genetics. Now, we envision the most promising opportunities for investigation provided by recent genomic tools and the development of sophisticated culturing techniques.

On one hand, the current and future availability of rotifer genome sequences (Flot *et al.*, 2013; Franch-Gras *et al.*, 2017a) are expected to revolutionize the field of evolutionary ecology studies in animals that are not genetic models (Declerck & Papakostas, 2017). Genome and transcriptome sequencing may also result in unprecedented advances in population genotyping and in the detection of genes related to any biological process of interest. As evidence of this potential, some studies have already been successful in identifying genes related to diapause (Denekamp *et al.*, 2009; 2011; Clark *et al.*, 2012), reproductive modes (Hanson *et al.*, 2013a; 2013b) and aging (Gribble & Mark Welch, 2017). The regulation of the asexual and sexual phases of cyclical parthenogenesis is addressable using these tools. Here, we call for the need to couple such molecular approaches with concurrent changes in physiology, behavior or life history for a complete understanding of adaptation.

On the other hand, the large population sizes and short generation times of rotifers are expected to allow the testing of evolutionary hypotheses in the laboratory (i.e., to control for confounding factors), a methodological approach that is impeded in other animals due to practical constraints. Experimental evolution has the potential to demonstrate evolution in action and to quantify the strength of natural selection against that of other evolutionary forces. We envision that among the tests of these hypotheses will be additional studies on the evolution of sex, speciation processes, and rapid evolution in eco-evolutionary dynamics (Fussmann *et al.*, 2007; Post & Palkovacs, 2009; Ellner *et al.*, 2013; Declerck & Papakostas, 2017). Potential also exists to combine laboratory results with resurrection ecology studies in natural populations.

Combining genomics and experimental evolution studies is also a promising avenue of research. Finding the genomic signature of rapid evolutionary adaptations may provide insights into why some traits evolve faster than others (Tarazona *et al.*, 2017). From our perspective, the application of these tools to rotifer research will allow the (re)formulating and testing of old and new hypotheses in the field of theoretical evolutionary ecology and population biology to continue the path opened by Professor M. R. Miracle.

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Zooplankton vertical migration in two Sahara lakes with contrasting biotic environments

Henri J. Dumont

Department of Biology, Limnology section, Ghent University, Belgium and Department of Ecology, Jinan University, Guangzhou, China.

* Corresponding author: henri.dumont@ugent.be

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ABSTRACT

Zooplankton vertical migration in two Sahara lakes with contrasting biotic environments

In two Saharan freshwater lakes (gueltas), 3-6 m deep, one with and one without fish, zooplankton migrated vertically. The guelta with fish had a very impoverished zooplankton, mainly composed of copepodids of cyclopoid copepods. They showed a strong diurnal migration. The guelta without fish had *Daphnia*, *Asplanchna*, and the fairy shrimp *Streptocephalus*. *Daphnia* migrated normally at first, but was later disturbed by *Streptocephalus* that moved around in swarms and physically interfered with the smaller zooplankton. Interference competition is a factor to be considered whenever several species of zooplankton migrate side by side. This may result in a vertical stratification of species, and in extreme cases, in reverse migration.

Key words: zooplankton, diurnal migration, predation, competition, *Streptocephalus*, *Asplanchna*, cyclopoids, Sahara

RESUMEN

Migración vertical del zooplankton en dos lagos del Sahara con entornos bióticos contrastantes

En dos lagos saharianos de agua dulce (guelta), de 3-6 m de profundidad, uno con y otro sin peces, se observó la migración vertical del zooplancton. La guelta con peces tenía un zooplancton muy empobrecido, compuesto principalmente de copépodos ciclopoideos. Mostraron una fuerte migración diurna. La guelta sin peces tuvo Daphnia*,* Asplanchna *y el camarón* Streptocephalus*. La* Daphnia *migró normalmente al principio, pero luego fue perturbada por el* Streptocephalus *que se movió alrededor de la guelta en grupos e interfirió físicamente con el zooplancton más pequeño. La competencia por interferencia es un factor por considerar cada vez que varias especies de zooplancton migran una al lado de la otra. Esto puede dar como resultado una estratificación vertical de las especies y, en casos extremos, una migración inversa.*

Palabras clave: *zooplancton, migración diaria, predación, competición,* Streptocephalus*,* Asplanchna*, ciclópidos, Sáhara*

INTRODUCTION

Diel vertical migration of the various size groups of the marine and freshwater zooplankton is possibly the most common and widespread animal migration on earth (Williamson *et al.*, 2011). That zooplankters swim to the surface during the night hours, and migrate down during the day has been known since the 19th century, and has generated hundreds of papers. One early review, still mechanistic in its approach, was by Cushing (1951). It was followed by many updates, such as Hutchinson (1967), Lampert (1989), Dumont & De Meester (1990), Ringelberg (2010) and others, in which the tone became more and more Darwinian. It has become customary to call factors that are under selection ultimate, whereas signals that initiate movements are called proximate. Some factors are both. Light, for example, is as well a signal for migration to begin (Ringelberg, 1999), as a fitness component, via damage done to tissues and DNA, especially by short wavelengths (Hairston, 1979). But light also influences predation pressure, especially by fish, on animals that fail to swim down to deep dark layers rapidly enough during daytime, may be a strong selection power (Zaret and Suffern, 1976; Gliwicz, 1986a; Lampert, 1989). Swimming to the surface too early may also be under selection, as even the light provided by the rise of the full moon has been found adequate to allow fish to locate and forage on crustacean zooplankters reaching the surface too early (Gliwicz, 1986b).

The Sahara is a place with abundant light but few freshwater lakes. Still, in appropriate, deeply eroded mountain environments, freshwaters of variable duration, with a depth of up to 12 m (although modally not more than 3-4 m) occur. They are locally called guelta (diminutive: gueltet). Partially related with depth are permanence and salinity, which depend on water renewal rate. Permanent freshwater gueltas usually have a variety of fish, non-permanent ones (even if so

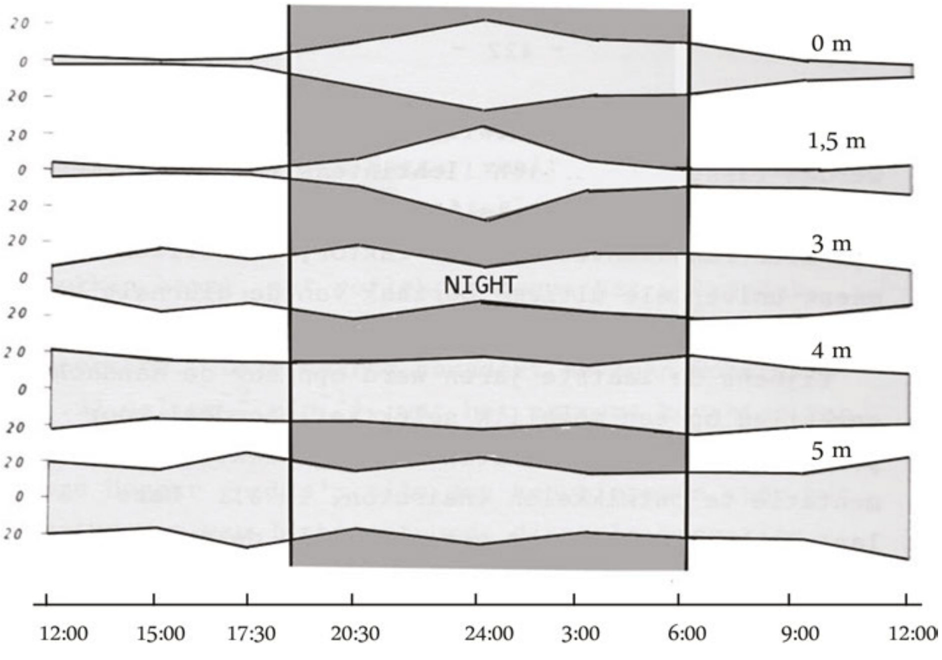

Figure 1. Diurnal migration of cyclopoids in Guelta Molomhar, Adrar, Mauretania. An outspoken upward movement occurred at the surface and down to 1.5 m during the night. Numbers here became extremely low during the day. Migration became insignificant below 3 m. *Migración diurna de ciclopoidos en Guelta Molomhar, Adrar, Mauritania. Un claro movimiento ascendente ocurrió en la superficie y hasta 1.5 m durante la noche. El número de ciclopoidos fue bastante bajo durante el día. La migración fue insignificante por debajo de los tres metros.*

deep that they fall dry only once per decade on average) are fishless.

Here, I comment on the results of two 24 hour cycles on Molomhar and Gueltet Zli, two gueltas in the Adrar, a mountainous area in western Mauretania, Western Sahara, in winter 1976.

MATERIAL AND METHODS

Molomhar (280 m a.s.l, 20° 35’ N, 13° 09’ W; annual precipitation at the nearby city of Atar 70 mm). is situated in a deep, narrow canyon with the same name. It has almost vertical walls, such that sampling down to 6 m could simply be done from a ledge. The guelta is elongated, with a length of about 100 m, and a width of not more than 3-5 m in most places. The water is rather transparent and has a green algal colour, but no exact measurements were taken. Its permanent nature is underscored by the presence of at least four species of fish, including the pelagic *Barbus deserti*, up to some 5 cm in size, and a zooplanktivore. Gueltet Zli (300 m a.s.l, 19° 31’ N, 12° 47’ W) is situated in a more open landscape, but –typical for most gueltas – lies at the foot of a waterfall that only discharges for few days, after rare torrential rainfall events. It was less than completely full at the time of our visit, but still had depths of over 3 m. The water transparency was higher than in Molomhar, but probably not to the point where it would affect the amplitude of migration (Dodson, 1990). Sampling was

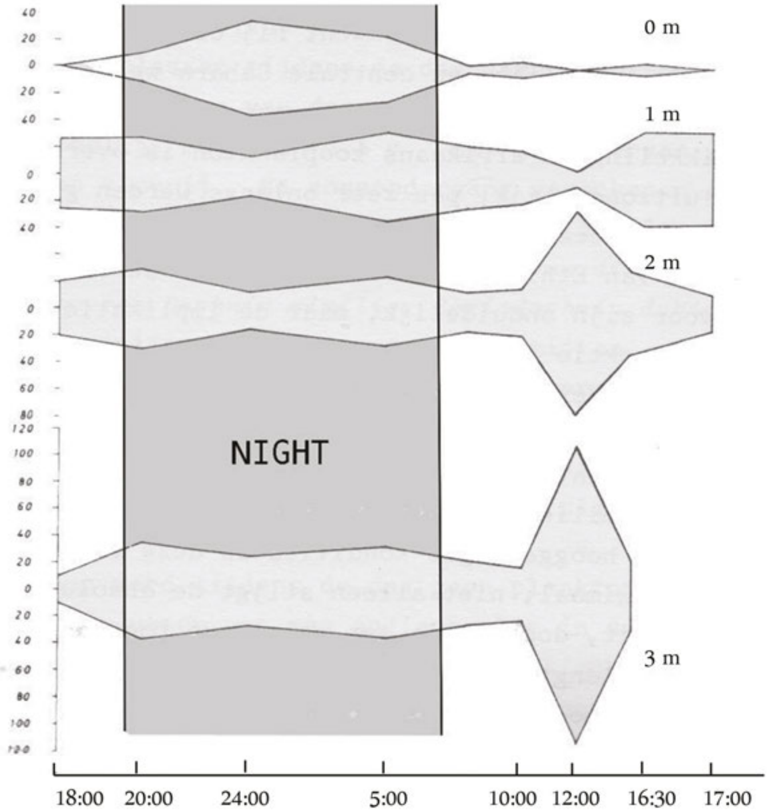

Figure 2. Diurnal migration of Daphnia similis in Gueltet Zli, Adrar, Mauretania. The daytime values at the surface during the day were almost nil but increased during the night. There is no clear evidence of migration in deeper strata, but patchiness occurred, especially at 2 and 3 m during the second day (last sample at 3 m was lost). *Migración diurna de Daphnia similis en Guelta Molomhar, Adrar, Mauritania. Los valores durante el día en la superficie fueron prácticamente nulos, pero aumentaron durante la noche. No hay evidencia clara de migración a mayor profundidad, pero hubo parches, especialmente durante el segundo día a 2 y 3 metros (la última muestra, a tres metros, se perdió).*

conducted from an inflatable boat, in the center of the lake, which had a diameter of about 50 m. There were no fish, but sizeable numbers of the filter-feeding fairy shrimp *Streptocephalus torvicornis* (Waga, 1948) could be seen moving around.

Plankton was collected by lowering a 2 liter Friedinger plankton trap to the desired depth, and filtering the contents over a 50 µm mesh plankton net. Samples were preserved in 4 % formalin. Samples were counted upon our return to Europe, under a WILD stereomicroscope.

Sampling for vertical migration was carried out for 24 hours on 30-31 January 1976 (Molomhar), and 8-9 February 1976 (Zli), at 3 hour intervals, at the surface, 1.5 m, 3 m, 4 m, and 5 m (Molomhar), and at the surface, 1 m, 2 m, and 3 m (Zli).

Water temperature, pH, and dissolved oxygen were also measured, and we collected large-volume plankton samples by towing a 50 µm plankton net across the gueltas, horizontally and vertically, for studying zooplankton taxonomical composition.

RESULTS

Both gueltas were of freshwater (Molomhar ca 200 µS, Zli 190 µS), and had an alkaline pH (7.8-8.8). Dissolved oxygen was mostly somewhat below saturation, more so in the enclosed Molomhar (61-84 %) than in the more open Gueltet Zli (86-102 %). Water temperature was around 20 °C in both gueltas, decreasing at the surface by 1-2 degrees during the night, and with little or no vertical stratification (about 0.5 degrees difference between surface and 6m).

Zooplankton in Molomhar was impoverished and composed of small individuals, all under 1 mm. I found rare specimens of *Diaphanosoma excisum* Sars, 1885 copepodids of *Mesocyclops* sp., *Thermocyclops* sp., and rotifers (*Brachionus* sp., *Keratella tropica* (Apstein, 1907), *Polyarthra* sp., *Asplanchna* sp.). Cyclopoid copepods were the only taxon numerous enough for further analysis. In counting, we lumped all copepodid stages.

At Gueltet Zli, one reasonably common rotifer was *Asplanchna priodonta* Gosse, 1850, beside the large cladoceran *Daphnia similis* Claus, 1876. The only copepod seen was *Metadiaptomus mauretanicus* Kiefer & Roy, 1942, but in low numbers, too few to be analyzed.

In Molomhar, I recorded a clear ’classical’ migration: the surface layers, down to 1m depth, were depleted of copepods during the day. Upwardly migrating specimens during the night seemed to be recruited from the 2 m level (Fig. 1). Overall, numbers were low, with a maximum of about 40 animals per liter.

At Gueltet Zli, *Daphnia similis* showed a similar upward migration during the night, and deserted the surface during the day of 9 February. During the day, there was also a sudden increase in abundance at the 2 and 3 m levels (Fig. 2), from around 40 individuals per liter to about 200.

Still in Gueltet Zli, *Asplanchna priodonta* can perhaps best be defined as not migrating at all, although by the second day, an overall increase in numbers below 1m depth occurred (Fig. 3).

DISCUSSION

It is clear that the patterns of migration between the two gueltas were different, and that these differences did not derive from any chemical of physical characteristics of the lakes involved.

In Molomhar, the zooplankton circadian rhythm was of the classical type, consistent as well with a light as with a fish predation driver. Probably, both causal factors acted in concert. This allows us to confirm the occurrence of diurnal vertical migration to an environment as extreme as the Sahara Desert.

In Gueltet Zli, *Daphnia* migrated normally, at least at the surface, in the first half of the cycle, but the pattern became disturbed during the last third. The reason for this was unexpected but could be observed by eye: it was the fairy shrimp *Streptocephalus* that moved around the guelta in one or more swarms. It avoided the Friedinger bottle and also escaped being captured by the towed plankton net. I hypothesize that the moving swarm(s) forced out the *Daphnia* and *Asplanchna* from their immediate vicinity, causing them to aggregate elsewhere, giving rise to contagious spatial distributions. Probably the fairy shrimps themselves migrated upwards during the night, as observed in laboratory conditions by Brendonck *et al.* (1995), adding to the disturbance. During

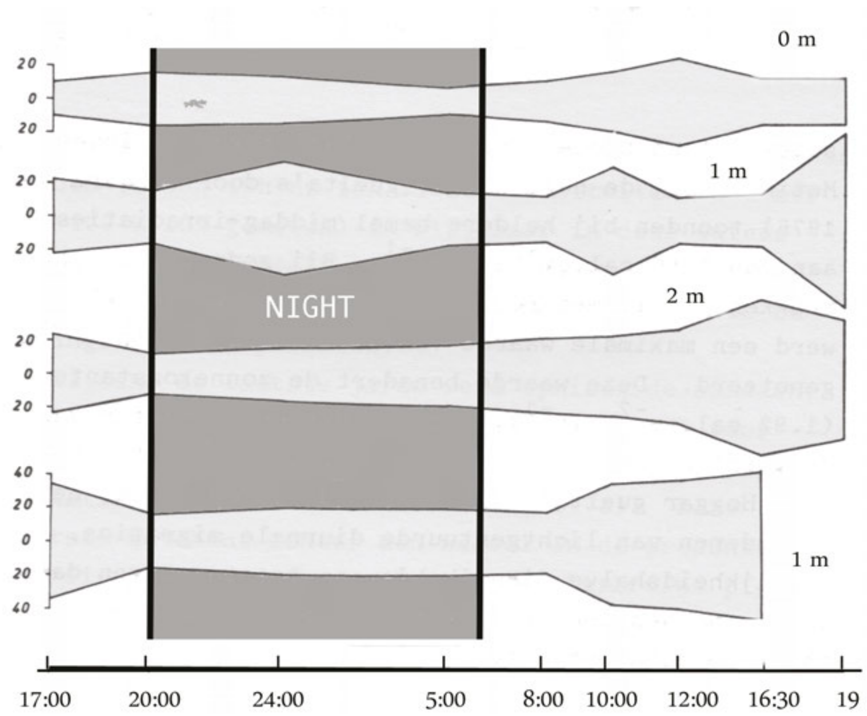

Figure 3. Diurnal migration of Asplanchna priodonta in Gueltet Zli, Adrar, Mauretania. No migration occurred at any depth level, but there was evidence of patchiness. Numbers here became extremely low during the day. Migration became insignificant below 3 m. *Migración diurna de Asplanchna priodonta en Guelta Molomhar, Adrar, Mauritania. No hubo migración a ninguna profundidad, pero hubo evidencia de parches.*

the day, when they could visually be observed, the swarms never came to the surface, remained below the 1 m level and preferentially stayed in the shadow of the rocky shores. Voucher specimens captured with a hand net lacked carotenoid colors, known to protect from UV damage. (Hairston, 1979; Rautio & Tartarotti, 2010). This suggests that adjusting their position to deeper or shadowed strata may have been these animals' main way to avoid excess UV radiation.

I stress that vertical migration was maintained in the absence of vertebrate predation. The main difference was that the abundance although probably not the species richness (see Gliwciz *et al.*, 2010, for a discussion of this phenomenon) in the plankton community increased in fishless conditions and that interference competition between the larger and smaller species occurred. The 3-4 cm long fairy shrimp have no protection against vertebrate predation and are mutually exclusive with fish. However, fairy shrimp have a natural tendency to form swarms, and may mechanically displace cladocerans and rotifers, causing their distribution to become patchy too and disturbing their migration pattern. This 'competition for elbow room' or avoidance of it by a different vertical incidence (Armengol & Miracle, 2000), especially clear in rotifers, seems akin to the potential conflict between *Asplanchna* and *Bosmina* in a shallow eutrophic lake at high numerical densities, where *Asplanchna* migrated inversely, while *Bosmina* and cyclopoids migrated normally (Dumont, 1972). Exploitative competition involving *Asplanchna* can be ruled out, as it is a pure carnivore that, being a jelly plankter, can be physically damaged by armored crustaceans. Such conflicts may also arise intraspecifically, as shown within a *Daphnia longispina* population migrating vertically in a Spanish lake (King & Miracle, 1995). Electrophoretically distinct subpopulations here displayed distinct movement types, perhaps similar to the genetically distinct phototactic clones isolated in the laboratory by Dumont *et al.* (1985) and De Meester (1991).

CONCLUSIONS

To the best of my knowledge, this is the first time zooplankton vertical migration has been shown to occur in the most extreme desert of the world. Selecting two lake lets with a different predation environment revealed that as well escape from visual predators as from excess light may elicit migration. High fish predation pressure, however, impoverishes the zooplankton community to the point that very few species (in the present case only one) survive, and that cladocerans can become eliminated.

Williamson *et al.* (2011) provided a detailed and interesting discussion of proximate and ultimate factors involved in vertical migration, but omitted one relevant variable: interactions between the migrating animals themselves. Inter- and intraspecies competition for space or food indeed might be much more common than currently appreciated in shaping particular migratory patterns on a variety of time scales.

ACKNOWLEDGEMENTS

I dedicate this paper to Maria Rosa Miracle, who was deeply interested in zooplankton vertical migration as well as in the Sahara. Seven months after the field work for the present paper, Maria joined me in field work in the central Saharan Hoggar mountains, where we visited and sampled six gueltas. She told me afterwards that this was one of the high points of her life. I am happy I could offer her this experience. My thanks also go to the participants in my second Sahara expedition, who helped with the sampling for this study: the late Miel Van Cappellen, Jo Vermeir, Daniel Somers, and Ionel Miron. The late Mokhtar Ould Bontemps guided us to the gueltas, which were suggested to me by my old mentor Theodore Monod.

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Rotifer communities in Mediterranean ponds in eastern Iberian Peninsula: abiotic and biotic factors defining pond types

Maria Sahuquillo[1,2,*] and Maria Rosa Miracle[1]

[1] Departament de Microbiologia i Ecologia, Institut Cavanilles de Biodiversitat i Biologia Evolutiva, Universitat de Valencia, E-46100 Burjassot, València, Spain.
[2] Subdirecció General del Medi Natural, Generalitat Valenciana, València, Spain.

[*] Corresponding author: maria.sahuquillo@uv.es

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ABSTRACT

Rotifer communities in Mediterranean ponds in eastern Iberian Peninsula: abiotic and biotic factors defining pond types

We examined the rotifer community composition in 130 freshwater Mediterranean ponds which included a wide range of limnological characteristics. The objective was to test the effectiveness of rotifer assemblages in the identification of different ponds types and the forcing factors that determine their distribution. Among the environmental variables analysed hydroperiod stands out as a major factor separating true temporary ponds from those with permanent water stability. Abiotic and biotic factors showed a different relative importance in the two groups of ponds. Among the temporary ponds, abiotic variables, such as arid climate, turbidity and short hydroperiod defined a special group called *argillotrophic*, in which *Rhinoglena frontalis* was found to be the most characteristic rotifer species. Those temporary ponds with short hydroperiods supported few species (mean species richness 4) and, in the main, planktonic species. Permanent ponds supported higher species richness (mean 13 and maximum 33 species) composed mainly of littoral species. The rotifer community of permanent ponds was found to be correlated with biotic factors, such as fish and macrophyte abundance. High macrophyte cover favours complex habitat heterogeneity and a wider range of feeding traits, ranging from algae-grazing species to carnivorous species (*Dicranophorus grandis* or *Cupelophagis vorax*). These results showed that rotifer communities are useful to provide a typology of ponds with a hierarchical order of factors structuring them ranging from abiotic to biotic factors. This is an example of Stress Gradient Hypothesis being more significant in the heterogeneous Mediterranean area, and which may indicate trends of changes related to the conservation status or the effects of global warming.

Key words: stress gradient hypothesis, biodiversity, *Rhinoglena*, hydroperiod, temporary ponds, zooplankton

RESUMEN

Comunidades de rotiferos en charcas Mediterráneas en el este de la península Ibérica: factores abióticos y bióticos que definen los diferentes tipos de charcas

Se han estudiado las comunidades de rotíferos en 130 charcas mediterráneos con gran amplitud de características limnológicas. El objetivo es comprobar la efectividad de estas comunidades en la identificación de diferentes tipos de charcas, así como lo factores que determinan esta distribución. Entre las variables ambientales analizadas, el hidroperíodo se destaca como factor principal, separando las charcas temporales de las permanentes con mayor estabilidad del medio. Posteriormente, factores abióticos y bióticos muestran una diferente importancia relativa dentro de cada uno de estos dos grupos de charcas. Entre las charcas temporales, factores abióticos, como clima árido, elevada turbidez e hidroperiodos cortos, definen un grupo especial de charcas temporales argilotróficas, en las que Rhinoglena frontalis *es la especie de rotífero más característica. Estas charcas temporales con hidroperiodo corto tiene pocas especies de rotíferos (4 de media) y generalmente planctónicas. Las charcas permanentes tienen mayor riqueza de especies (13 de media 13 y un máximo de 33 especies) y más especies litorales. Estas comunidades de rotíferos de charcas permanentes están más relacionadas con factores bióticos, como la* *abundancia de peces y macrófitos. La elevada cobertura de macrófitos favorece una heterogeneidad del hábitat y una mayor gama de formas de alimentación, desde herbívoros hasta especies carnívoras (*Dicranophorus grandis *o* Cupelophagis vorax*). Estos resultados muestran que las comunidades de rotíferos son útiles para proporcionar una tipología de charcas y que existe un orden jerárquico de factores que las estructuran desde factores abióticos hasta factores bióticos. Un ejemplo de la hipótesis de gradientes de estrés, más significativa en la heterogénea área mediterránea, y que puede indicar la direccionalidad de cambios relacionados con el estado de conservación o los efectos del calentamiento global.*

Palabras clave: *hipótesis de los gradientes de stress, biodiversidad,* Rhinoglena*, hydroperiodo, charcas temporales, zooplancton*

INTRODUCTION

Several studies have recently addressed the biodiversity of ponds, the factors driving different biological communities, and the utility of using community structure to define different pond typologies (Indermuehle *et al.*, 2008; Céréghino *et al.*, 2008; Miracle *et al.*, 2010). While much work has been done on larger invertebrates and plants, rotifers, in spite of being an important component of the aquatic community has been neglected and less studied than other biological groups.

Rotifers are essential elements of freshwater ecosystems. They are important components of food webs due to their extremely high reproductive rates and their large populations (Walz, 1995). Sometimes they comprise the most important proportion of the zooplankton biomass (Wallace *et al.*, 2006). Rotifers are often the most preferred prey for larger invertebrates such as copepods (Lapesa *et al.*, 2002; Brandl, 2005) or small planktivorous fishes (Telesh, 1993). Their special biological characteristics make rotifers a model organism for ecological and evolutionary studies (Snell, 2014; Serra *et al.*, in press). Moreover, they are a diverse group comprising about 2000 species (Segers, 2007) and are ubiquitous components of aquatic biocenosis. Rotifers have high dispersal abilities, are widely distributed in all types of inland waters and therefore generally thought to be cosmopolitan. However most rotifer species have their own biogeographical distribution pattern (Dumont, 1983) and include very specialised fauna which are sensitive to environmental changes (Angeler *et al.*, 2010). Environmental variables identified to have significant effects on rotifer diversity include temperature, salinity or aquatic vegetation (Malekzadeh Viayeh & Spoljar, 2012; Duggan *et al.*, 2001; Guisande *et al.*, 2008; Kaya *et al.*, 2010). Sometimes these factors depend on the scale of the study. For example, within a single pond there are spatial differences between the open waters and the littoral area, or even species-specific preferences for microhabitats due to different types of macrophytes stands (Celewicz-Gołdyn & Kuczyńska-Kippen, 2017). Differences in the trophic state, salinity or hydroperiod arise among groups of ponds (Mazuelos *et al.*, 1993; Duggan *et al.*, 2002; Wallace *et al.*, 2005; Serrano & Fahd, 2005). Expanding the analysis from a local to a regional level would provide additional insights of how climatic and biogeographical factors influence rotifer distribution (Duggan *et al.*, 2002).

The Mediterranean basin shares common factors but it is considered a heterogeneous area. The diverse climate, geology and topography and the intense and long human footprint has resulted in a rich patchwork of habitats and landscapes (Blondel *et al.*, 2010). One common feature is the irregular and scarce precipitation. In Mediterranean regions where water is scarce, small-sized aquatic habitats are abundant and extremely important due to their social and economic value as well as their ecological role in biodiversity. The maintenance of a good conservation status of these aquatic systems requires an adequate functioning of all their components. A good knowledge of every necessary step of their complex structure will provide further tools for their maintenance.

Our work was undertaken to study rotifer assemblages in Mediterranean ponds in the eastern Iberian Peninsula. We analysed 130 ponds arranged along a climatic and hydrological gradient. The accuracy of identifications and the high number of ponds studied helped improve our knowledge of the diversity of rotifers in the region. The main objectives of this study were: (1) to test the effectiveness of rotifer assemblages

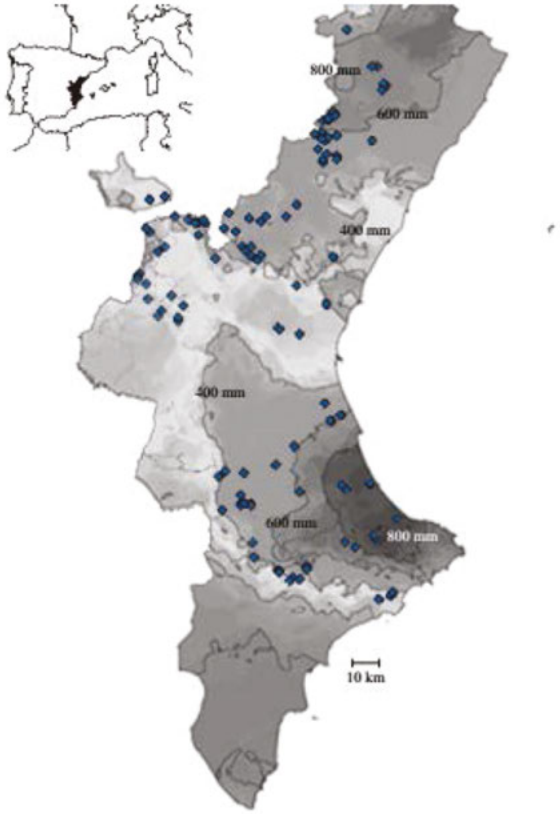

Figure 1. Geographical location of the 130 Mediterranean ponds studied in Comunitat Valenciana, eastern Spain. The isohyets show mean annual precipitation based on data for a 30-year reference period. Darker shading indicates higher precipitation. *Localización de las 130 charcas mediterráneas estudiadas en la Comunitat Valenciana. Las isoyetas muestran la precipitación media anual para un periodo de referencia de 30 años. Las zonas más oscuras corresponden a una mayor precipitación.*

in the identification of different ponds types; (2) to identify environmental and water features that shape these communities; (3) to analyse differences in rotifer assemblages characteristic of the pond types, and; (4) to contribute to the knowledge of the biodiversity of rotifers, a neglected taxa in small ponds in the Mediterranean area.

METHODS

The study area

Locations of the ponds studied are shown in figure 1. The study area has a typical Mediterranean climate with variations in rainfall from semi-arid (mean annual rainfall 300 mm/year) to sub-humid (above 800 mm/year), and in temperature from lowland warm areas to cooler mountains (mean annual temperature 17 and 9 ºC respectively, Pérez Cueva, 1994). Small ponds are frequent in the study area (Sancho & Lacomba, 2010) and include diverse environmental characteristics. The 130 ponds included in this study were all freshwater, shallow (0.2 – 4.0 m depth) and small. The area of 50 % of the studied ponds was < 200 m^2 (only 4 % > 2000 m^2). They cover a broad range of hydroperiod, from ephemeral rain-fed ponds to permanent ponds fed by groundwater. Thus, they constitute an appropriate dataset for investigating the influence of graded hydrological variation and pond limnology on rotifer composition. Physical and chemical characteristics of these ponds and some faunal communities were described in previous works (Sancho & Lacomba, 2010; Sahuquillo & Miracle, 2010; Sahuquillo *et al.*, 2012; Sahuquillo & Miracle, 2013 and Sahuquillo & Miracle, 2015).

Data collection

We sampled ponds in 2006, 2007 and a few in 2008. Sampling was performed between February and early-May, to avoid early and late wet phases in the temporary ponds. Most ponds were sampled once but about 20 % were sampled twice (in two different years to provide samples from two late winter–early spring periods). Rotifers were exhaustively sampled in littoral and open waters usually between 10:00 and 16:00. Semi-qualitative pelagic samples were taken using 40 µm meshed plankton net in horizontal hauls. In littoral vegetated areas we used a 60 µm handnet. Quantitative samples were obtained by filtering through a 30 µm mesh between 4 – 16 L of depth-integrated water samples with a transparent tube (1 or 0.5 m long and 5 cm internal diameter) in different parts of the water column. Zooplankton samples were preserved in 4 % formaline. For quantitative samples all specimens were identified and counted with an inverted microscope at 100x and results were expressed as individuals per litre. For net samples we counted sub-samples to obtain relative abundances, until

Table 1. Categories of pond, based on hydroperiod, used in this study. *Categorías de charcas, en función del hidroperiodo, utilizadas en este estudio.*

Pond type based on hydroperiod		Definition
Temporary short hydroperiod ponds	TS	< 3 months
Temporary intermediate hydroperiod ponds	TM	from 3 to 6 months
Temporary long hydroperiod ponds	TL	> 6 months but with annual summer drying
Semi-permanent ponds	SP	dry every few years
Permanent ponds fed by groundwater	P	permanent

no statistical variation was observed or no other new species were found. Rotifer species were identified using Koste (1978). Rotifer carbon contents as mass were taken from Latja & Salonen (1978), Telesh *et al.* (1998) and Lehtovaara & Sarvala (unpublished) and converted to dry weight using a ratio of 2.5.

Morphological (depth, area) and biological characteristics of ponds (plant coverage) were estimated *in situ*. In the central open water area we measured *in situ* water temperature, dissolved oxygen, conductivity and pH using WTW probes and we took a depth integrated sample of the water column with a transparent tube (as described above) for laboratory analysis of turbidity, alkalinity, planktonic chlorophyll-*a*, total phosphorus, total nitrogen and ammonium, following APHA (1992). We assigned a categorical variable to each pond indicating the main source of water (1 = rainfall, 2 = surface waters and 3 = ground waters) and one of five hydroperiod categories described in Table 1 based on our own observations. For each pond, several climatic parameters related to annual and spring rainfall, temperature and climate indices for a 30-year reference period (1960–1990) were obtained from the nearest meteorological station in Pérez Cueva (1994). More comprehensive information, including data on crustacean community composition and physical and chemical characteristics of water and sediments of the same ponds and pools were previously published in Sahuquillo & Miracle (2012 and 2013). In fact, in this study we also tried to determine whether rotifer communities responded to environmental factors as crustaceans do in the same ponds, as we reported in Sahuquillo & Miracle (2013).

Data analysis

1.- To find a pond typology we used multivariate analyses. Due to the large number of rotifer taxa identified, for these analyses we reduced the number of variables by grouping some taxa at the genus level. To obtain a representative composite sample for each pond, the relative percentages of rotifer taxa were averaged for each pond and date, these relative percentages were arcsine square root transformed to normalise the data. We conducted an initial ordination of rotifer taxa and ponds by detrended correspondence analysis (DCA) to check for differences in rotifer community composition and to estimate the species gradient length. DCA analysis was performed using the program Multivariate Statistical Package (MVSP 3.0 for Windows). Multivariate regression tree analysis (MRT), was used to look for (i) a hierarchical ordination of the variables affecting rotifer composition; (ii) a threshold value (cut-off value) of those variables that function as predictors and (iii) clusters of sites ('leaves'). The splits were chosen to minimise the Bray–Curtis dissimilarity index within clusters, and each split is defined by a simple rule based on environmental variables (De'Ath & Fabricius, 2000; De'Ath, 2002). Predictive accuracy was estimated from the cross-validated relative error (CVRE), and CVRE minimum was used as the size of the best predictive tree ('pruned tree', De'Ath, 2002). MRT analyses were computed with the program R (Package mvpart version 1.2-6).

2.- In order to explore the differences in patterns of biodiversity among the different pond types, the following indices and estimators were calculated. Species richness was estimated as the total number of rotifer taxa recorded in each pond at each date (local species richness –or point species richness, Magurran, 2004). Differences in species richness, diversity and rotifer biomass among the different pond types are presented in box-plots with median and percentiles. Assumptions of normality and equal variance of data were checked prior to analysis and data were log or power transformed to improve normality of variables. ANOVA and post hoc Tukey tests for unequal sample sizes (Unequal N HSD) were applied to transformed variables to test for significant differences among pond types. Analyses were performed using PAST Statistics Software Package (Hammer *et al.*, 2001)

The most characteristic species for each final pond type was selected from those having the highest indicator value INDVAL (Dufrêne & Legendre, 1997) and with a significance of taxa association to typology $P < 0.01$. This index is a measure to express species importance in community classifications. The analysis takes into account the relative abundance and frequency of occurrence of each taxon within the pond type and was performed using the software PC-ORD for Windows, 4.20, Oregon.

RESULTS

During this study a total of 109 monogonont rotifer taxa belonging to 35 genera were differentiated, of which 95 were identified to species (Table 2). The genus *Lecane* was, by far, the most diverse with 24 species, followed by *Brachionus* and *Trichocerca* with 7 identified species each. Bdelloids were also frequent in the ponds studied. In temporary ponds the rotifer community was dominated by planktonic species whilst plant-associated rotifers were the predominant population of permanent ponds.

Rotifer ordinations

A first ordination of ponds and rotifer taxa through DCA analyses showed the studied ponds ordered in a continuous gradient from more ephemeral ponds (TS) on the left side, to permanent ones (P) on the right one (Fig. 2). Among the rotifer species, the planktonic genera *Rhinoglena*, followed by *Filina*, *Hexarthra* and *Brachionus* reached the lowest values on this axis and were associated with temporary ponds with short to medium hydroperiod. On the opposite side, many species, mainly living on the littoral vegetated areas (*Colurella* sp., *Cupelopagis vorax*, *Dicranophorus grandis*, etc.), obtained high values on this axis and represent the more permanent ponds. Most samples were distributed along this first axis, while the second axis separated on the left those temporary ponds with a very short hydroperiod from those with a larger one (at the top of the graph). However hydroperiod had a lower effect on the right where more permanent ponds were grouped together. The results of the DCA ordination suggest an important role of hydroperiod on rotifer composition in the ponds.

MRT analysis, forcing factors

In a second stage, we applied MRT analysis to a matrix containing rotifer taxa and samples used for DCA analysis together with all limnological and climatic variables gathered. A first MRT analysis gave a two-leaf tree with the smallest estimated predictive error (CV = 0.64) (Fig. 3). This first split had the maximum drop in deviance (that is, the longest lengths of the vertical line segments), and further splits had lower importance. The first split was based on hydroperiod. This primary split separated the ponds into two groups: on the left are the permanent (P) and semi-permanent ponds (SP) together with temporary ponds with long hydroperiod (TL), whilst on the right are temporary ponds with medium or short hydroperiod (TM and TS).

We also ran an un-pruned tree analysis to observe subsequent splits. Within the group of more permanent ponds, a second split separated at one side (leaf 1) permanent ponds (P) with fish and mainly fed by groundwater. On the other side, semi-permanent ponds (SP) remained together with temporary ponds with long hydroperiod (TL). Among this last group of ponds, the plant coverage was a variable selected to subsequently

Table 2. List of rotifer taxa and percentage of occurrence in temporary (TS = temporary ponds with short hydroperiod, TM=medium hydroperiod, TL with long hydroperiod), semipermanent (SP) and permanent (P) study ponds. *Lista de especies de rotíferos y porcentaje de ocurrencia en las charcas estudiadas. TS = charcas temporales con hidroperiodo corto; TM = hidroperiodo medio; TL = temporales con hidroperiodo largo); S= semi-permanentes; P = permanentes.*

TAXA	TS	TM	TL	SP	P
Bdelloidea	42	39	33	78	80
Anuraeopsis fissa Gosse*,* 1851	9	2		4	7
Ascomorpha ecaudis Perty*,* 1850					5
Asplanchna sp.	2			4	9
Asplanchnopus hyalinus Harring, 1913					4
Asplanchnopus multiceps (Schrank, 1793)					2
Brachionus angularis Gosse, 1851	4				5
Brachionus variabilis Hempel, 1896					4
Brachionus quadridentatus Hermann, 1783	7		67	2	4
Brachionus urceolaris Müller, 1773	15	18			4
Brachionus plicatilis Müller, 1786					2
Brachionus calyciflorus Pallas, 1766					4
Cephalodella gibba (Ehrenberg, 1830)	2	2		2	25
Cephalodella catellina (Müller, 1786)					4
Cephalodella sp.	24	48	83	37	40
Colurella adriatica (Ehrenberg, 1831)	2			7	33
Colurella colurus (Ehrenberg, 1830)				4	15
Colurella hindenburgi Steinecke, 1916					2
Colurella obtusa Gosse, 1886	4	7		9	18
Colurella uncinata (Müller, 1773)				7	11
Collotheca sp.		5	17	2	
Cupelopagis vorax (Leidy, 1857)				4	18
Dicranophorus grandis (Ehrenberg, 1832)					35
Euchlanis dapidula Parise, 1966					4
Euchlanis dilatata Ehrenberg, 1832	2	16	50	24	56
Encentrum martoides Fott, 1960					2
Encentrum plicatum Harring & Myers, 1928					2
Encentrum longipes Wulfert, 1936					2
Epiphanes brachionus (Ehrenberg, 1837)		2			
Eosphora najas Ehrenberg, 1830					7
Filinia longiseta (Ehrenberg, 1834)	25	25		2	2
Floscularia sp.				2	
Hexarthra mira (Hudson, 1871)	27	48		13	
Hexarthra intermedia (Wiszniewski, 1929)	4		17		
Hexarthra sp.		2	17	4	2
Keratella cochlearis (Gosse, 1851)				2	
Keratella tropica (Apstein, 1907)	2	2	67	11	18
Keratella procurva (Thorpe, 1891)	7			13	
Keratella quadrata (Müller, 1786)	7	16	33	9	
Lecane bulla (Gosse, 1851)	11	18		15	49
Lecane aculeata (Jakubski, 1912)				2	15
Lecane bifurca (Bryce, 1892)					4
Lecane closterocerca (Schmarda, 1859)	11	23	17	26	36
Lecane cornuta (Müller, 1786)					5
Lecane curvicornis (Murray, 1913)					2
Lecane decipiens (Murray, 1913)					2
Lecane doryssa Harring, 1914					2
Lecane flexilis (Gosse, 1886)				11	
Lecane furcata (Murray, 1913)	2				
Lecane grandis (Murray, 1913)					4
Lecane hamata (Stokes, 1896)	2		33	7	35
Lecane hastata (Murray, 1913)	2			2	4

Cont.

Table 2. (cont.)

Lecane hornemanni (Ehrenberg, 1834)					2
Lecane inermis (Bryce, 1892)					4
Lecane inopinata Harring & Myers, 1926					4
Lecane levistyla (Olofsson, 1917)					2
Lecane ludwigii (Eckstein, 1883)					4
Lecane luna (Müller, 1776)	11	18	17	30	51
Lecane lunaris (Ehrenberg, 1832)	11	18	17	35	33
Lecane mira (Murray, 1913)				2	
Lecane papuana (Murray, 1913)					5
Lecane punctata (Murray, 1913)				2	
Lecane pyriformis (Daday, 1905)					27
Lecane quadridentata (Ehrenberg, 1830)					33
Lecane scutata (Harring & Myers, 1926)					2
Lecane ungulata (Gosse, 1887)					9
Lecane sp.	2	5		2	13
Lepadella (*Lepadella*) *patella* (Müller, 1773)	4	41	67	17	49
Lepadella (*Lepadella*) *triptera* (Ehrenberg, 1832)		25	33	2	4
Lepadella (*Lepadella*) *ovalis* (Müller, 1786)	5	5	17	39	13
Lepadella (*Lepadella*) *rhomboides* (Gosse, 1886)		5		2	18
Lepadella (*Lepadella*) *acuminata* (Ehrenberg, 1834)					2
Lepadella sp.			17	2	2
Lophocharis salpina (Ehrenberg, 1834)				7	13
Monommata aequalis (Ehrenberg, 1830)	2	2		4	
Monommata actices Myers, 1930					2
Monommata sp.					13
Mytilina mucronata (Müller, 1773)	2	23		2	2
Mytilina ventralis (Ehrenberg, 1830)				4	7
Notholca acuminata (Ehrenberg, 1832)					20
Notholca squamula (Müller, 1786)		11		2	9
Notholca sp.				7	
Notommata copeus Ehrenberg, 1834				4	22
Notommata haueri Wulfert, 1939					2
Polyarthra longiremis Carlin, 1943	4		17	4	
Polyarthra vulgaris Carlin, 1943					4
Polyarthra sp.	20	14		17	13
Paradicranophorus sp.					2
Ptygura melicerta Ehrenberg, 1832					5
Ptygura sp.			33		
Rhinoglena frontalis Ehrenberg, 1853	27	18			
Scaridium longicaudatum Ehrenberg, 1830				4	4
Squatinella mutica Ehrenberg, 1832				2	
Squatinella rostrum (Schmarda, 1846)					29
Synchaeta oblonga Ehrenberg, 1832					18
Synchaeta pectinata Ehrenberg, 1832					4
Synchaeta kitina Rousselet, 1902					2
Synchaeta sp.		7		11	29
Testudinella patina (Hermann, 1783)	27	45	17	28	29
Trichocerca pusilla (Jennings, 1903)	24	25		9	2
Trichocerca elongata (Gosse, 1886)	15	36	17	9	
Trichocerca rousseleti (Voigt, 1902)	4				
Trichocerca rattus (Müller, 1776)				2	25
Trichocerca elongata (Gosse, 1886)					9
Trichocerca longiseta (Schrank, 1802)					11
Trichocerca porcellus (Gosse, 1851)					25
Trichocerca sp.	16	7	17	13	11
Trichotria pocillum (Müller, 1776)				15	31
Trichotria tetractis (Ehrenberg, 1830)				7	15
Tripleuchlanis plicata (Levander, 1894)					5

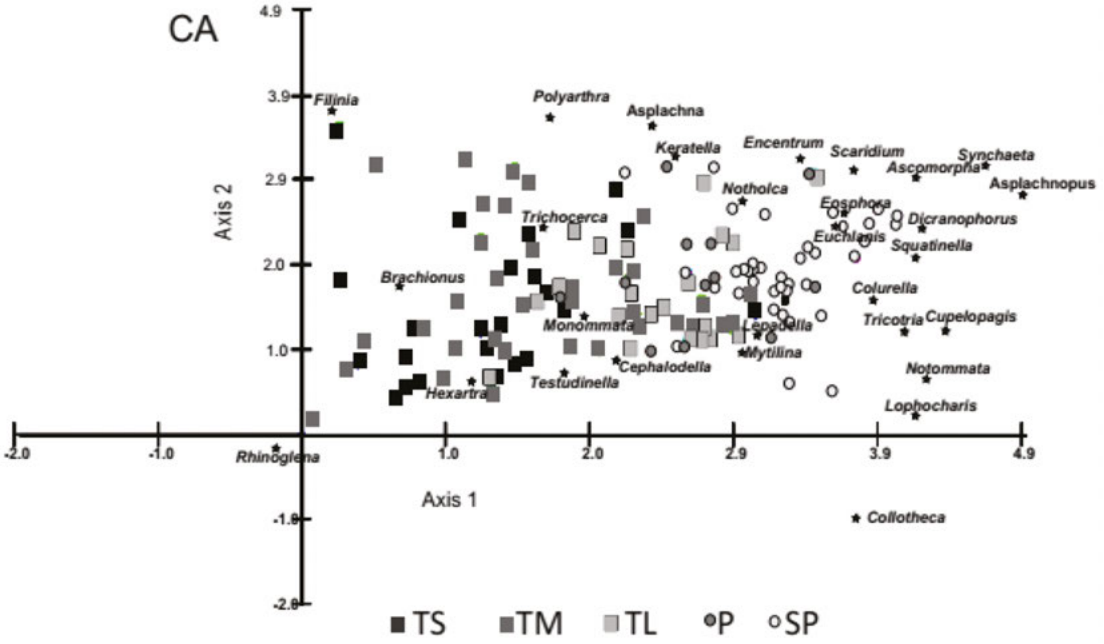

Figure 2. Biplot of samples (squares and circles) and species (stars) resulting from DCA ordination. Samples were classified according with hydroperiod classes used in this study: TS, black squares, Temporary short hydroperiod pools; TM, dark grey squares, Temporary intermediate hydroperiod pools; TL, light grey, Temporary long hydroperiod pools; SP dark grey circles, Semi-permanent ponds and P, light circles, permanent ponds. *Biplot mostrando la ordenación de muestras (cuadrados y círculos) y especies (estrellas) resultantes del análisis DCA. Las muestras se clasificaron de acuerdo con las clases de charcas en función del hidroperiodo utilizadas en este estudio: TS, cuadrados negros, charcas temporales de hidroperíodo corto; TM, cuadrados grises oscuros, charcas de hidroperiodo intermedio temporal; TL, gris claro, charcas temporales de hidroperiodo largo; SP círculos gris oscuro, balsas semi-permanentes y P, círculos blancos, balsas permanentes.*

differentiate rotifer communities in a group of ponds with macrophytes (leaf 2) from another group of SP and TL, with low plant coverage.

In the third split, the temporary ponds with short and medium hydroperiod (TS and TM) were separated into two groups. In this split, turbidity, spring precipitation and plant coverage were the selected variables, and defined a first group of ponds (leaf 4) located in wetter areas (with spring mean precipitation higher than 156 mm) associated with clear waters and plant coverage higher than 15 % of pond bottom. On the other side (leaf 5), those temporary ponds in more arid areas with clay turbid waters and low macrophyte coverage remain.

We then looked for a correspondence between the five leaves of ponds obtained from the MRT analyses and the ponds included previously in the categories based on hydroperiod (Table 1). We found a high correlation in the groups: leaf 1 corresponds to permanent ponds fed by groundwater (P); leaf 4 corresponds to temporary intermediate hydroperiod pools (TM) and leaf 5 corresponds to temporary short hydroperiod pools (TS). However, the leaves 2 and 3 do not fit well with established categories and temporary long hydroperiod pools (TL) and semi-permanent ponds (SP) were grouped together.

Patterns of diversity among pond types

Species richness varied considerably among the pond types, with an increasing trend along the gradient of water permanency (Table 2; Fig. 4) although the statistical test among different pond types, only became significantly higher in permanent ponds. Shallow temporary ponds with shorter hydroperiod had the fewest number of rotifer species, with a mean of 4 species per pond and date. However, a few temporary ponds had local

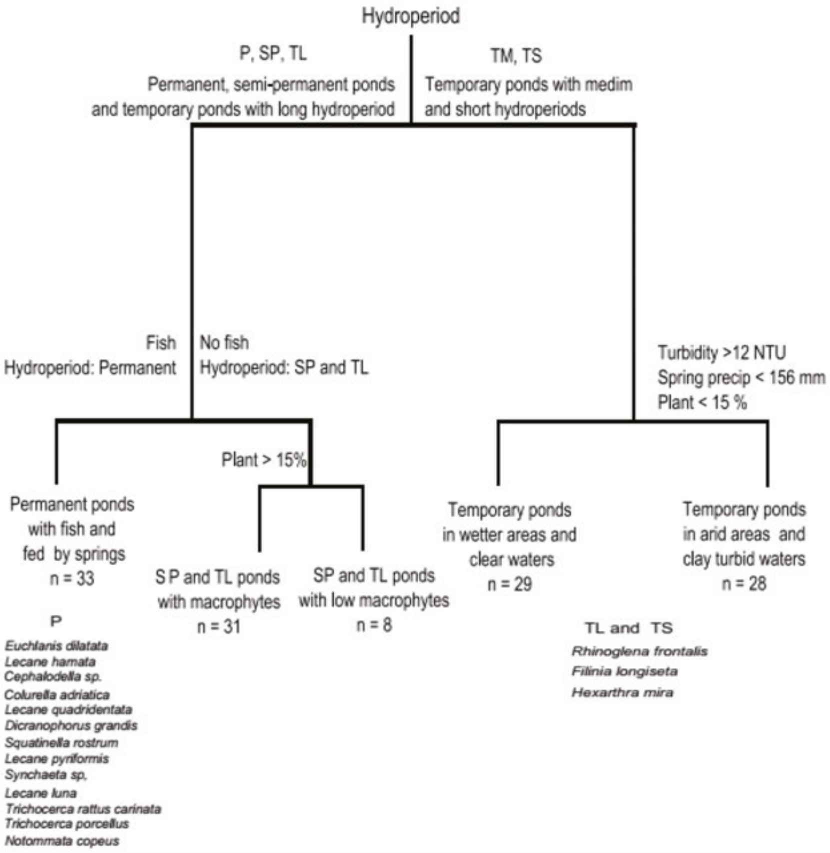

Figure 3. Multivariate regression tree (MRT) based on rotifer community composition from 130 ponds. The lengths of the vertical line segments are proportional to the drop in deviance corresponding to each split and the labels for each node show the variables selected for the split and the thresholds. n = the number of studied ponds for each pond type. Abundant taxa with INDVAL scores > 60 are shown at the bottom. *Árbol de regresión multivariante (MRT) basado en la composición de la comunidad de rotíferos de 130 charcas estudiadas. Las etiquetas para cada nodo muestran las variables seleccionadas para la división y sus umbrales. n = el número de charcas en cada tipo. Los taxones con valor INDVAL > 60 se muestran en la parte inferior.*

species richness above 10 taxa, a number comparable with those from permanent ponds. The number of taxa increased slightly as the observed hydroperiod lengthened. At the opposite extreme, the group of permanent ponds had higher species richness, averaging 13 rotifer species and with a maximum of 33 taxa. Not surprisingly, the ponds with the greatest species richness were two clear permanent ponds fed by groundwater with dense macrophyte cover (named Fosc and Rajolar). These differences are even less clear when we look at Shannon diversity indices. The group of ponds fed by groundwater (P) presented maximum values of diversity. Nevertheless, among the other groups diversity values were similar to each other. Although we observed a certain tendency towards increased diversity with more stable aquatic habitats, differences were not statistically significant. Rotifer biomass in plankton samples was low in the more permanent ponds (groups P and TL), but quite variable within the rest of the groups. The low biomass was due to low density of rotifers. In temporary ponds, although very variable we can found very high rotifer biomasses in plankton samples.

INDVAL

In temporary ponds only three taxa reached INDVAL scores higher than 60, therefore indicat-

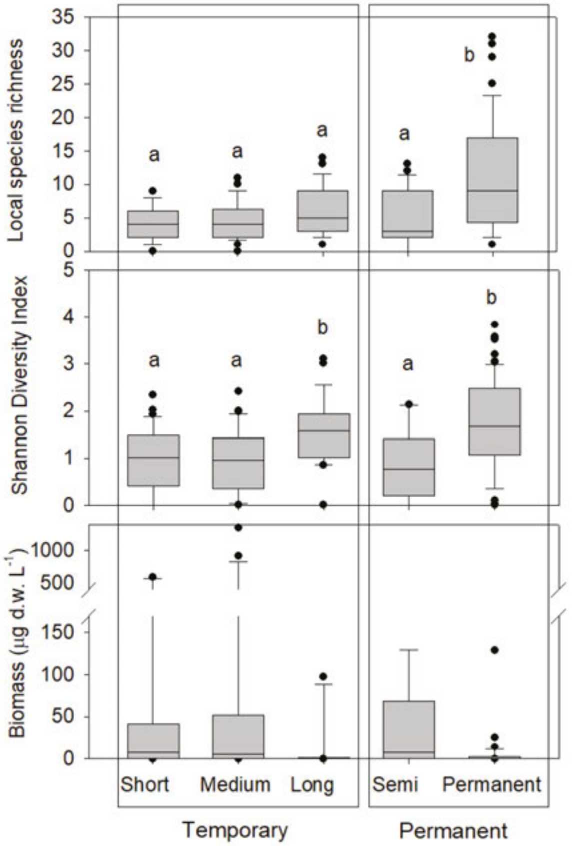

Figure 4. Boxplots showing several diversity estimators for each pond type: Local species richness was calculated for individual samples (i.e., per pond and sampling date), Shannon diversity index and rotifer biomass. *Diagramas de caja mostrando los resultados de diversos estimadores de diversidad calculados para los diferentes tipos de charcas estudiados: riqueza local de especies (por charca y día de muestreo), índices de diversidad de Shannon y biomasa de rotíferos. Las letras sobre los gráficos indican los grupos significativamente diferentes (pruebas post-hoc de Tukey).*

ing that these are typical species for this pond type. The low number of indicator species was expected as the total number of species per pond was also very low. These were all planktonic species. *Rhinoglena frontalis* reached highest INDVAL values in temporary ponds with short or medium hydroperiod. This species was quite frequent in our samples; we found it in 23 out of 35 temporary ponds (Table 2). Other species with high INDVAL values in temporary ponds with medium and short hydroperiod were *Filinia longiseta* and *Hexarthra mira*.

On the contrary, in the more permanent ponds with fish, a high number of species, 13 taxa, have significant INDVAL scores (Fig. 3), including some rare species such as *Dicranophorus grandis*, *Squatinella rostrum*, *Lecane pyriformis* or *Notommata copeus*. Most species found in these ponds have littoral preferences.

DISCUSSION

The extensive survey of ponds in a large variety of habitats allowed us to find a pond typology based on rotifer community. In addition these pond types also differ in the main structuring factors, in species richness, rotifer feeding traits and habitat preferences. Among the analysed environmental variables included in the studied freshwater ponds, hydroperiod was the environmental factor most strongly associated with the distribution of rotifer species. Although salinity has been identified as a crucial factor in determining rotifer abundance and diversity (Hammer, 1986), we analysed only freshwater ponds. Consequently, hydroperiod stands out as a major factor separating true temporary ponds from those with permanent water stability, as many studies have shown (Williams, 1998; Serrano & Fahd, 2005; Marrone *et al.*, 2006; Boix *et al.*, 2007; Della Bella *et al.*, 2008; Sahuquillo & Miracle, 2013 and many others). Subsequently, abiotic and biotic factors showed different relative importance within the groups of temporary and permanent ponds.

Within temporary ponds, significant environmental variables which explain variability in rotifer communities include both abiotic (rainfall and clay turbidity) and biotic (macrophyte cover) factors. The abiotic variables highlight the role of water scarcity which define “harsh” habitats with very short hydroperiod and clay turbid waters. These *argillotrophic* temporary ponds are frequently found in Mediterranean arid areas and harbour very specialised taxa (Marrone *et al.*, 2006). In our case, *Rhinoglena frontalis* was found to be the most characteristic rotifer species in this pond type. The apparent importance of inorganic turbidity explaining the distribution of *Rhinoglena* (MRT analyses showed that this is an important factor) may indicate that food plays a large role. Clay turbid water implies low primary producers and some fauna must be specialized in order to feed on bacteria and organic matter adsorbed in the suspended clay particles. *Rhinoglena* populations reached very high densities in plankton samples and so it may also play an important role as food for higher trophic aquatic inhabitants. Additionally, *Rhinoglena* was always found when temperatures were very low in winter and with clay turbid water. This is a widespread cold stenothermal species (Koste, 1978; Segers, 2007) which typically inhabits temporary habitats (Schröder, 2005). In our study area it was quite frequent in temporary ponds (23 ponds) with low water temperatures (preferably 5 – 10 ºC) and low conductivity (75 – 500 µS/cm). Amazingly, this species was not found in other extensive studies of temporary ponds in the Iberian Peninsula (De Manuel, 1992 in Balearic islands and Guisande *et al.*, 2008 or Serrano & Fahd, 2005 in Doñana). In our study this species was found in cold mountain ponds and temperature could therefore be the limiting factor explaining this distribution. Those temporary ponds with short hydroperiods support few species (mean = 4). The number of taxa per pond was quite similar to those observed in Doñana ponds (Serrano & Fahd, 2005) or in semi-arid ponds in Iran (Malekzadeh Viayeh & Spoljar, 2012).

Within the permanent pond types, with more stable habitats, the rotifer community was found to be correlated with biotic factors, such as fish and macrophyte abundance. Most of the rotifer species found had littoral preferences. In these permanent ponds, with structurally complex habitats (high macrophyte cover) more refuges reduce predator foraging efficiency and also competition may increase diversity through increased habitat specialization (Menge & Sutherland, 1976). Higher macrophyte cover supports higher habitat heterogeneity, food availability and refuge in a more stable environment and can also support more rotifer species. Not only the macrophyte density (Cyr & Downing, 1988), but also the morphological structure influence zooplankton assemblages (Kuzinska-Kippen & Nagengast, 2006). Moreover, competition favours a wider range of feeding traits, from algae-grazing species to carnivorous species. This is the case of the predatory rotifer *Dicranophorus grandis* and the littoral/sessile carnivorous *Cupelophagis vorax*. On the contrary, few cases of carnivorous rotifers have been documented in temporary ponds (Walsh *et al.*, 2014). Permanent ponds as a whole held a higher number of species (120) than temporary ones, and local species richness was also higher (mean 13 and maximum 33 species). Not surprisingly, some permanent ponds with clear waters and high macrophyte cover sustain extremely high species richness in spite of their small area. Similar richness values were previously found in these habitats (Alfonso & Miracle, 1987; Miracle *et al.*, 1995) and these values are comparable to the ones obtained in larger water ecosystems considered to have high rotifer diversities. We consider these high richness values to result from a high sampling effort, great spatial habitat diversity, high habitat temporal stability and a good ecological status.

This hierarchical order of factors structuring these rotifer communities were also found for crustaceans in the same ponds (Sahuquillo & Miracle, 2013). It is a good example of the importance of physical environment controlling organisms in harsh environmental conditions whilst biological interactions are more significant in more benign and predictable conditions (Lévêque, 1997). Many studies support this stress gradient hypothesis (SGH; Callaway, 2007) and perhaps it could be more significant in the heterogeneous Mediterranean area.

With respect to the main objectives of this study we found that rotifer communities can be used to classify ponds with contrasting limnological and climatic characteristics. First abiotic factors, and then biotic factors were identified to have a significant role on rotifer community distribution. Also, the results of this study contribute to the knowledge of rotifer fauna which has not been studied before in these ponds and showed the extraordinary richness of the rotifer assemblages in these environments. Traditionally, estimates of aquatic biodiversity have focused on permanent waters, however, temporary waters can make significant contributions to rotifer species richness and harbour high levels of endemism (Galindo *et al.*, 1994; Walsh *et al.*, 2014). We are far from fully understanding this fauna and we recognize that our study lacked more intensive seasonal sampling and /or interannual variability. In order to adequately manage and preserve these unique aquatic habitats, additional work will be needed to assess their biodiversity and to understand the processes regulating it.

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Macroecological and spatial patterns in the distribution of cladocerans in Alpine lakes

Fabio Stoch[1,*], Daria Vagaggini[2] and Fiorenza Gabriella Margaritora[2]

[1] Evolutionary Biology & Ecology, Université Libre de Bruxelles, Avenue F.D. Roosevelt 50, B-1050 Brussels, Belgium.
[2] Sapienza University of Rome, Department of Environmental Biology, Viale dell'Università 32, I-00185 Rome, Italy.

[*] Corresponding author: fabio.stoch@ulb.ac.be

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ABSTRACT

Macroecological and spatial patterns in the distribution of cladocerans in Alpine lakes

An extensive data set of the occurrence of 55 cladoceran species in 432 Alpine lakes was used to document patterns of species richness, assemblage composition and distribution across the Alpine ecoregion in Italy, determining the spatial and macroecological parameters explaining the observed patterns. Moreover, the influence of dispersal, environmental filtering and life cycle strategies in shaping cladoceran alpha and beta-diversity patterns was examined. Only three species where widely distributed in the Alpine lakes, with more than 80 % of the species present in less than 10 % of the lakes. Alpha-diversity ranged from 1 to 13 species, while beta-diversity was almost exclusively due to spatial turnover. Our data suggest that the excess of rarity in these environments was better explained by post-glacial random recolonization patterns, high dispersal ability, peculiar life strategies including the production of diapausing eggs, and compliance of cladocerans to the monopolization hypothesis. Spatial determinants (studied using Moran's Eigenvector Maps) were important for large scale species distribution patterns, suggesting that dispersal was not a limiting factor, although altitudinal heterogeneity shaped beta-diversity patterns. The altitudinal gradient (together with the associated macroclimatic factors) was the second main determinant of species distribution, followed by the geological setting of the basins. Local factors played only a minor role in explaining cladoceran distribution patterns at the ecoregional scale.

Key words: Lakes, large-scale patterns, beta-diversity, rarity, logarithmic distribution, monopolization hypothesis

RESUMEN

Patrones espaciales y macroecológicos en la distribución de cladóceros en lagos alpinos

Una extensa lista de 55 especies de cladóceros de 432 lagos alpinos fue utilizada para estudiar los patrones de riqueza, composición y distribución de especies a lo largo de la ecorregión alpina en Italia, determinando las variables espaciales y macroecológicas que explican los patrones observados. Además, se examinó el efecto de la dispersión, del filtrado ambiental y de las estrategias en el ciclo de vida, en los patrones de diversidad alfa y beta en cladóceros. Solamente tres especies estuvieron ampliamente distribuidas en los lagos Alpinos, con más del 80 % de las especies presentes en menos del 10 % de los lagos. La diversidad alfa, varió entre una y trece especies, mientras que la diversidad beta fue casi exclusivamente debida al recambio en el espacio. Nuestros datos sugieren que la rareza en estos ambientes estuvo mejor explicada por patrones aleatorios de recolonización post-glaciar, alta habilidad de dispersión, estrategias de vida peculiares, incluyendo la producción de huevos diapáusicos y la adaptación de los cladóceros a la hipótesis de monopolización. Las variables espaciales (estudiadas con Moran's Eigenvector Maps) fueron importantes para los patrones de distribución de las especies a gran escala, sugiriendo que la dispersión no fue un factor limitante a pesar de la heterogeneidad altitudinal relacionada con los patrones de diversidad beta. El gradiente altitudinal (junto con los factores macroclimáticos asociados) fue el segundo determinante en la distribución de las especies, seguido por la geología de la cuenca. Los factores locales tuvieron poca *importancia al explicar los patrones de distribución de los cladóceros a escala ecorregional.*

Palabras clave: *Lagos, patrones a gran escala, diversidad beta, rareza, distribución logarítmica, hipótesis de monopolización*

INTRODUCTION

Evaluating how different groups of species vary along environmental gradients can provide valuable information on the relative importance of spatial and ecological drivers (Marquet *et al.*, 2004; Pinel-Alloul *et al.*, 2013). Notably, climatic conditions have been often invoked, given that temperature, precipitation and productivity are well correlated with species richness patterns in zooplankton (Hawkins *et al.*, 2003). Moreover, historical hypotheses have also been suggested for formerly glaciated areas, where dispersal processes, influenced by environmental barriers and the presence of local glacial refuges, may have had effects on present-day species richness and distribution patterns (Henriques-Silva *et al.*, 2016). Freshwater microcrustaceans are good candidates to test these hypotheses (Leibold *et al.*, 2010); however, different mating strategies and different life history cycles suggest that Allee effects, i.e. the decrease in the average fitness of individuals in low-density populations, influence the relative importance of environmental filtering and dispersal limitation on species diversity patterns (Courchamp *et al.*, 1999). For this reason, Henriques-Silva *et al.* (2016) suggested that lumping species with large differences in their susceptibility to Allee effects, like copepods and cladocerans, should be avoided in examining large-scale patterns.

Cladocerans living in Alpine lakes were selected in our study as the target group to document macroecological patterns. Cladocerans were considered good candidates for several reasons: they reproduce almost invariably through cyclic parthenogenesis (De Meester *et al.*, 2002), and, for this reason, should be less subject to the Allee effect than copepods (Henriques-Silva *et al.*, 2016). Moreover, cladocerans are a key group of organisms in many Alpine lakes, comprising an important component of the zooplankton and benthos, and their distribution is well known, especially in the Italian Alps (Tonolli & Tonolli, 1951; Giussani *et al.*, 1986). The Alpine chain was subject to Quaternary glaciations and re-colonized from local refugia ("nunatakker", e.g. nunatak theory: Dahl, 1987) as well as southern ice-free areas. This well defined ecoregion (Blasi *et al.*, 2014) is highly heterogeneous, shows a strong altitudinal gradient likely to influence species distribution, and a high degree of naturalness. Due to the interaction of historical, ecological and spatial factors, the Alps constitute the ideal scenario to document the role of spatial and ecological determinants shaping cladoceran distribution patterns.

Already in the late 19th and early 20th century, several Alpine lakes were studied and their zooplanktonic fauna described in detail (Zschokke, 1900, 1908; Pesta, 1929). Zschokke (1900) observed no relationship between species richness and altitude, but found that lakes located on granitic substrates generally had more diverse fauna than lakes located in dolomitic areas, suggesting the importance of geological settlement of basins as a further species diversity determinant. More recently, some studies indicated that microcrustacean species composition, including cladoceran assemblages, may be changing in alpine lakes especially due to the introduction of fish (Guilizzoni *et al.*, 2006 and literature cited therein). However, Winder *et al.* (2001) resampled lakes studied in the early 20th century in Switzerland, concluding that species richness significantly decreased with increasing lake altitude, but no other environmental factor correlated with richness. Consequently, the presence of fish was categorically excluded as a determinant of the absence of large zooplankton (especially *Daphnia*) in Alpine lakes.

Therefore, although local environmental factors are still claimed as the major determinants of cladoceran species richness and distribution patterns in lakes compared to regional-scale factors (Leibold *et al.*, 2010), the debate is still open (Anas *et al.*, 2014; Pinel-Alloul *et al.*, 2013), and the role of pure spatial factors, as potential

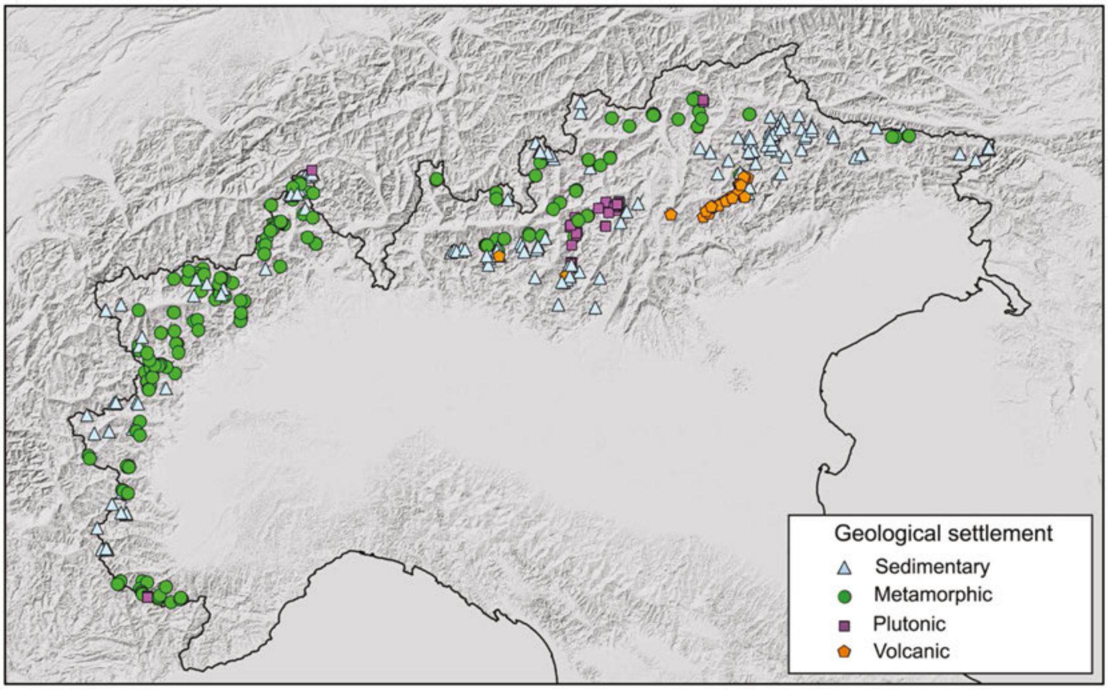

Figure 1. Geographical distribution of the 432 studied lakes and their geological settlement along the Italian Alps. *Distribución geográfica de los 432 lagos estudiados y su asentamiento geológico a lo largo de los Alpes italianos.*

surrogates of cladoceran dispersal ability, probably underestimated (Sweetman *et al.*, 2010).

The aims of our study, based on an extensive data set of cladoceran distribution in 432 Alpine lakes in Italy, are threefold: (i) document for the first time the patterns of species richness, assemblage composition and distribution of lake cladocerans across the Alpine ecoregion; (ii) determine the spatial and ecological determinants explaining the observed geographical distribution patterns, and (iii) discuss the role of dispersal and environmental filtering in shaping cladoceran diversity patterns at the study ecoregional scale.

MATERIAL AND METHODS

Study area

The Alps are the highest and most extensive mountain range system in Europe, stretching about 1200 kilometers across eight countries. The Alpine ecoregion in Italy is a well-defined combination of structural, climatic and biogeographic features (Blasi *et al.*, 2014). In our study the geographical boundaries are those defined by SOIUSA (an Italian acronym for 'International Standardized Mountain Subdivision of the Alps'), the most recent classification system of the Alps (Marazzi, 2005). SOIUSA main classificatory system divides the Alps in 2 main parts (PT: Western and Eastern Alps), 5 sectors (SR), 36 sections (SZ). The sections are useful geographic units for general national reporting and are adopted in our study as main operational units.

Sampling methods and bibliographical review

The large-scale surveys published by Tonolli & Tonolli (1951) and Giussani *et al.* (1986), the monograph of the Italian cladocerans (Margaritora, 1985) as well as the database of cladoceran distribution in Italy produced by Margaritora (2005), updated to 2017 with new literature and collection records, were used as starting points for compiling the species distribution matrix used in the analyses. A database of the native cladocerans reported to occur in the Alpine ecoregion was compiled from these sources after an extensive and critical literature review (see Supplementary information, available at http://www.limnetica.net/en/limnetica).

An extensive, integrative sampling survey was carried out (1990–2014) on 108 lakes widespread along the whole Alpine arc in Italy. In each lake two samples were collected in July-August. Zooplankton samples were collected in the deepest point of the lakes, when possible with the aid of a small inflatable boat. The plankton net (mouth 25 cm diameter, 60 µm mesh size) was towed vertically from the bottom to the surface. Littoral samples were taken using the same net or a hand-net (semicircular mouth 25 cm diameter, same mesh size) along the shoreline. Sample fixation in 4 % formaldehyde or 96° ethanol (especially useful for large-bodied Daphniidae) was carried out in the field to avoid damage to body shape and the morphological characters used for identification. Lake morphometric parameters (maximum depth and area) were measured in the field, together with selected limnological parameters (water temperature and conductivity); the presence of fish was annotated. Samples were sorted under a stereoscopic microscope (20X - 60X) and cladocerans separated from detritus, labelled and stored in 75° alcohol for conservation in small vials. Identification was carried out under a compound microscope by FGM. Cladoceran identifications were based on Margaritora (2005), with the exception of those taxa which changed rank or were synonymized after the publication of this reference work (Adamowicz & Purvis, 2005; Van Damme *et al.*, 2010). Samples were deposited in the collection of "La Specola" Museum of the University of Florence.

Considering the uncertain status of cladoceran subspecies (especially in the genera *Daphnia* and *Ceriodaphnia*), they were not considered as separate units when compiling the occurrence matrix. As regards the genus *Daphnia*, molecular methods applied to the *Daphnia longispina* (O. F. Müller, 1776) species complex, widespread on the Alps, suggested that some well-distinguishable morphospecies (like *D. hyalina* Leydig, 1860, *D. zschokkei* Stingelin, 1894 and *D. rosea* Sars, 1862) should be considered as ecotypes of *D. longispina* (Petrusek *et al.*, 2008). The widespread establishment of clonal populations (Haileselasie *et al.*, 2016) makes the interpretation of the results of molecular studies quite difficult. Pending a thorough revision of these taxa, a conservative approach, according to the recent monograph of Błedzki & Rybak (2016), was followed, and the updated nomenclature of Fauna Europaea (https://fauna-eu.org) was adopted herein.

Lakes to be included in the species distribution (presence/absence) matrix were selected based on reliable literature records and newly collected samples; only permanent lakes were selected avoiding the inclusion of temporary lakes which can host a peculiar cladoceran fauna; lakes located at an altitude below 1000 m a.s.l. were discarded. A total of 432 lakes (Fig. 1) were retained for the analyses (see Supplementary information, available at http://www.limnetica.net/en/limnetica).

Regional and local environmental data

Coordinates of the barycenters of the 432 lakes were obtained both in the field, checking published literature or newly georeferencing lakes using 1:25 000 topographic maps available at the National Cartographic Portal (http://www.pcn.minambiente.it). All the coordinates taken by GPS or published in the literature where carefully verified using Google Earth (http://earth.google.com); precision of georeferencing was about 1 m. Lake area (SUP) was calculated using the tools available in Google Earth and in the National Cartographic Portal. Three sets of regional variables (spatial, geological, and climatic variables) were obtained for each lake and for each geographical area.

Spatial variables. Considering that longitude and latitude alone were unsatisfactory descriptors of spatial relationships along the Alpine arc (which shows a S-N extension in the western part, and a W-E extension in the central and eastern part) as well as of the irregular distribution pattern of sampled lakes, distance-based Moran's Eigenvector Maps (dbMEMs: Borcard & Legendre, 2002; Legendre & Legendre, 2012) were used to describe the spatial structure of the dataset. dbMEMs represent a spectral decomposition of the spatial relationships, producing orthogonal eigenvectors which were used to represent spatial relationships among sites. The eigenvectors with higher eigenvalues usually describe broad spatial structures, encompassing the spatial variation in the whole study area, while the last positive eigenvectors (with lower eigenvalues) describe finer spatial structures (Dray *et al.*, 2012). Moreover, spatial factors can be interpreted also as surrogates of dispersal processes (Stoch *et al.*, 2016), allowing to gain a better insight into the importance of dispersal as a determinant of cladoceran distribution patterns. Moran's Eigenvectors Maps were computed using R freeware software 3.3.2 (R Development Core Team, 2016), package ‘spacemakeR’ (Dray, 2008) from a truncated geodesic distance matrix between lake centroids obtained with R package ‘fields’ (Furrer *et al.*, 2011). The longest distance connecting two regions in the minimum spanning tree was used as a threshold to truncate the distance matrix. The dbMEMs extracted by the analysis which showed significant correlation with the species distribution matrix (large scale spatial variables: dbMEMs 1, 2, 3; medium scale: dbMEMs 8, 9, and 38) are reported in the Supplementary information, available at http://www.limnetica.net/en/limnetica. The same analysis applied to the Alpine areas used lake-areas centroids and area itself was used as a further spatial variable.

Geological variables. Geological data were obtained for each lake and (as coverage) for each region from the lithological map of Italy (available at http://www.pcn.minambiente.it) at 1:250 000 scale. Five main geological categories were used: plutonic (PLU), volcanic (VOL), metamorphic (MET), sedimentary (non-dolomitic, SED) and dolomitic (DOL) rocks (Fig. 1); dummy variables (1/0) were used to include the geological information in the site environmental matrix (one of the variables, VOL, was thus excluded from the analysis, being uninformative having all the values equal to zero).

Climatic variables. The considered environmental variables, besides region area (A), were those representing key aspects of macroclimate and topography. Climatic and topographic variables were obtained from WorldClim 1.4 layers (Hijmans *et al.*, 2005). Temperature and precipitation values are 1950–2000 means or ranges, interpolated to a 30’’ resolution (Hijmans *et al.*, 2005). The following variables were used: mean annual temperature (Tm); maximum temperature of warmest month (Tmax); minimum temperature of coldest month (Tmin); mean annual precipitation (Pm); precipitation of driest quarter (Pdq); precipitation of wettest quarter (Pwq). Actual evapotranspiration (AET) represent 1920–1980 annual means at 30’’ resolution. AET provides a synthetic index of water–energy dynamics (O’Brien, 2006), which subsumes ambient energy and water availability, two crucial factors determining lake hydrological cycles and productivity. Data on AET were extracted from the world map released by Trabucco & Zomer (2010). Direct Normal Irradiation (DNI, 1 km resolution), i.e. the solar radiation component that directly reaches the surface [kWh/m^2] was extracted from the layer of the Global Solar Atlas website (Europe and Central Asia), available at http://globalsolaratlas.info/downloads/europe-and-central-asia. Altitude (ALT) and altitudinal range (ALTr), two of the main controls of climatic heterogeneity, were calculated based on the 20 m resolution DEM available from the National Cartographic Portal (http://www.pcn.minambiente.it). Point values or mean and range for each SOIUSA section of environmental variables were calculated using QGIS freeware software version 2.18 (QGIS Development Team, 2018).

Local variables. For a subset of 199 lakes (Giussani *et al.*, 1986), some additional local variables were available for each lake i.e. lake depth, Summer water conductivity (measured in July-August), presence/absence of fish, artificial/natural origin of the basins.

Statistical analysis of species diversity

Point species richness (i.e. alpha-diversity) was defined as the number of cladoceran species recorded in a single lake. Alpha-diversity statistical distribution was fitted using EasyFit software (www.math-wave.com). Relationships between species richness and the main environmental parameters were assessed by means of generalized additive models (GAM) to account for curvilinear relationships. All statistical analyses were performed with R software. Considering the results of GAM did not differ from that of ordinary least squares (OLS) using log-transformed species richness data, more user-friendly OLS results were used.

Occurrence of crustacean species across the Alpine ecoregion was illustrated by a rank-frequency diagram. The statistical distribution of the number of lakes where each species was present was fitted using EasyFit software as above.

Regional species richness was defined as the number of species contained in each SOIUSA section. Because sampling effort varied among regions, the first-order jackknife estimator of species richness was calculated (Pinel-Alloul *et al.*, 2013): Sj = Sobs + r (n–1)/n, where Sobs is the number of species observed in n lakes and r is the number of species present in one lake. A weak correlation between Sj and regional area was detected; however, correcting species richness values for regional area did not improve further analyses. Relationship between regional species richness and selected environmental parameters was assessed using ordinary least squares (OLS) in R.

Overall beta diversity for the whole Alpine ecoregion was measured using $ß_{sor}$ (Sorensen's index of dissimilarity) and its components $ß_{sim}$ (differences in composition caused by species turnover) and $ß_{nes}$ (differences caused by species losses or gains, i.e. nestedness) as implemented by Baselga (2010). A pairwise dissimilarity matrix based on $ß_{sim}$ was computed using command beta.pair in R package 'betapart' (Baselga & Orme, 2012). Agglomerative hierarchical clustering analysis based on the $ß_{sim}$ dissimilarity matrix, using command hclust in R package 'stats' with average linkage, was used in order to identify groups of regions with similar fauna.

The significance of dividing the Alpine ecoregion into the SOIUSA section based on the $ß_{sor}$ similarity matrix was assessed by means of Analysis of Similarities (ANOSIM: Clarke, 1993) using the R package 'vegan' (Oksanen *et al.*, 2017). The same analysis was also used to assess

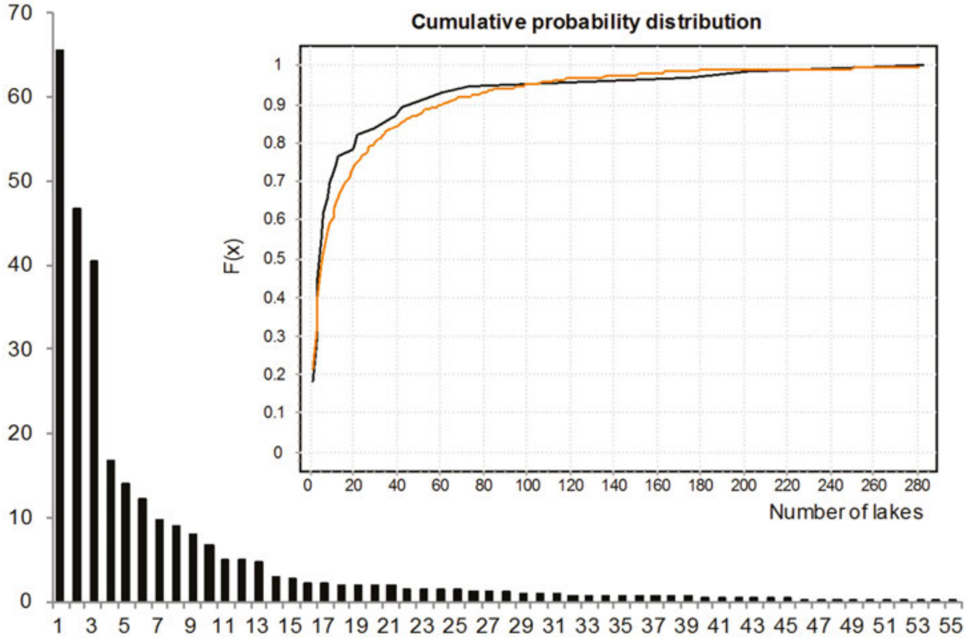

Figure 2. Rank frequency distribution of the 55 cladoceran species found in the studied Alpine lakes. The cumulative probability distribution illustrating the fit with a logarithmic distribution is reported as well. *Distribución de la frecuencia de rango de las 55 especies de cladóceros encontradas en los lagos alpinos estudiados. También se presenta la distribución de la probabilidad acumulada y el ajuste con una distribución logarítmica.*

the significance of cutting the $ß_{sim}$ - based tree into n clusters; this use has to be taken with caution, because the underlying argument is circular. For this reason, we merely inspected the results for site groups that were well-distinguished. A non-Metric Multidimensional Scaling analysis was performed in the R package 'vegan' based on the original species distribution matrix to visually inspect the consistence of the classificatory scheme from cluster analysis.

Statistical analysis of species distribution patterns and their determinants

Spatial and environmental predictors of species distribution patterns were assessed by a distance-based redundancy analysis (dbRDA: Legendre & Anderson, 1999) using command dbrda in R vegan package. dbRDA was used because it can be computed with any dissimilarity index and, therefore, $ß_{sor}$ could be preserved in the constrained ordination. Collinearity among variables was tested using command vif.cca in R package 'vegan'. After removing highly collinear variables (VIF > 20), a forward selection procedure with double-stopping criteria (Legendre & Legendre, 2012) was performed to avoid overfitting in R package 'packfor' (Dray, 2009), allowing to select only those variables that significantly explained the variation in the species dissimilarity matrix.

dbRDA analysis was applied three times to the dissimilarity matrices calculated on the following species distribution data sets: (i) species distribution matrix in lakes (55 species x 432 lakes); (ii) species distribution matrix of a subset of lakes where local environmental parameters were available (35 species x 199 lakes); (iii) species frequencies in the SOIUSA sections (55 species x 13 sections).

RESULTS

Cladoceran species richness and assemblage composition

Among the 55 cladoceran species recorded in the 432 Alpine lakes, the most diversified families were the Chydoridae (21 species) and the Daphniidae (18 species), which together accounted for 71 % of the total species richness, followed by the Macrothricidae (6 species). The other families included 1-2 species each (Table 1). Three species (*Chydorus sphaericus*, *Daphnia longispina* and *Alona affinis*) were found in more than 40 % of the lakes, and further three species of Chydoridae (*Alonella excisa*, *Alona quadrangularis*, and *Acroperus harpae*) were found in more than 10 % of the lakes. More than 80 % of the species were present in less than 10 % of the sampled lakes, while ten species were collected from a single lake.

The plot of the rank-frequency occurrences of the 55 cladoceran species (Fig. 2) was highly skewed. The best fit distribution was a logarithmic series (Anderson-Darling test, $p < 0.01$), with parameter $\theta = 0.9905$, which described very well the rarity pattern of Cladocera in Alpine lakes.

The efficiency of sampling effort in assessing Alpine cladoceran species richness was studied using sample-based rarefaction curves and different non-parametric estimators of species richness (Fig. 3). The Chao2 and ICE estimators converged between 300 and 350 sampled lakes, while the number of uniques (i.e. the number of species found in a single lake) decreased already after approximately 120 sampled lakes, indicating an efficient sampling effort. The species rarefaction curve as well as its estimators continued to increase slowly due to the high number of rare species evidenced by the rank-frequency occurrence. The discrepancy between measured and estimated species richness was around 6 species, i.e. 11 % of the assessed richness using estimators.

The average local species richness (alpha-diversity) per lake in the whole Alpine region ranged from 1 to 13 species (mean ± standard deviation = 3 ± 2, median 2). The best fit distribution was a Poisson distribution (Anderson-Darling test, $p < 0.01$), with parameter $\lambda = 2.8495$. The expected decrease in local species richness with altitude was confirmed for cladoceran assemblages; the strength of the relationship between log-transformed lake species richness and lake altitude was assessed using linear regression ($r = -0.24$, $p < 0.0001$, $n = 432$). Similar significant relationships ($p < 0.001$) were detected between local species richness and the climatic factors associated with altitude, like AET (r =

Table 1. List of the 55 cladoceran species reported from Alpine lakes in Italy; acronyms used in figures, number of lakes where present and frequency are reported. *Lista de las 55 especies de cladóceros reportadas en los lagos alpinos en Italia. Se indican los acrónimos usados en las figuras, número de lagos en los que hubo presencia y las frecuencias.*

Classification	Species	Acronym	Number of lakes	Frequency
Ctenopoda				
Sididae	*Diaphanosoma brachyurum* (Liévin, 1848)	Diabra	8	1.9
	Sida crystallina (O.F.Müller, 1776)	Sidcri	2	0.5
Holopediidae	*Holopedium gibberum* Zaddach,1855	Holgib	6	1.4
Anomopoda				
Bosminidae	*Bosmina (Bosmina) longirostris* (O.F.Müller, 1785)	Boslgr	35	8.1
	Bosmina (Eubosmina) coregoni (Baird,1857)	Boscor	1	0.2
	Bosmina (Eubosmina) longispina (Leydig, 1860)	Boslsp	10	2.3
Daphniidae	*Daphnia (Daphnia) cucullata* G.O.Sars, 1862	Dapcuc	2	0.5
	Daphnia (Daphnia) galeata G.O.Sars, 1864	Dapgal	1	0.2
	Daphnia (Daphnia) hyalina Leydig, 1860	Daphya	12	2.8
	Daphnia (Daphnia) longispina O.F.Müller, 1785	Daplon	202	46.8
	Daphnia (Daphnia) middendorffiana Fischer, 1851	Dapmid	8	1.9
	Daphnia (Daphnia) obtusa Kurz,1874	Dapobt	6	1.4
	Daphnia (Daphnia) rosea G.O.Sars, 1862	Dapros	13	3.0
	Daphnia (Daphnia) zschokkei Stingelin,1894	Dapzsch	22	5.1
	Ceriodaphnia dubia Richard, 1894	Cerdub	2	0.5
	Ceriodaphnia laticaudata P.E. Müller, 1867	Cerlat	3	0.7
	Ceriodaphnia pulchella G.O.Sars, 1862	Cerpul	5	1.2
	Ceriodaphnia quadrangula (O.F.Müller, 1785)	Cerqua	21	4.9
	Ceriodaphnia reticulata (Jurine,1820)	Cerret	5	1.2
	Ceriodaphnia rotunda G.O.Sars, 1862	Cerrot	1	0.2
	Ceriodaphnia setosa Matile, 1890	Cerset	1	0.2
	Scapholeberis mucronata (O.F.Müller, 1785)	Scamuc	10	2.3
	Simocephalus exspinosus (Koch,1841)	Simexs	2	0.5
	Simocephalus vetulus (O.F. Müller, 1776)	Simvet	39	9.0
Moinidae	*Moina brachiata* (Jurine, 1820)	Moibra	3	0.7
Ilyocryptidae	*Ilyocryptus sordidus* (Liévin, 1848)	Ilysor	1	0.2
Macrothricidae	*Drepanothrix dentata* (Eurén, 1861)	Drepden	3	0.7
	Lathonura rectirostris (O.F.Müller, 1785)	Latrec	1	0.2
	Macrothrix hirsuticornis Norman & Brady, 1867	Machir	42	9.7
	Macrothrix laticornis (Jurine,1820)	Maclat	4	0.9
	Streblocerus serricaudatus (Fischer,1849)	Strser	9	2.1
Eurycercidae	*Eurycercus lamellatus* (O.F.Müller, 1785)	Eurlam	6	1.4
Chydoridae	*Chydorus gibbus* Sars,1890	Chygib	2	0.5
	Chydorus ovalis Kurz,1874	Chyova	6	1.4
	Chydorus sphaericus O.F.Müller, 1785	Chysph	283	65.5
	Coronatella rectangula (G.O.Sars 1892)	Correc	29	6.7
	Acroperus angustatus G.O.Sars,1864	Acroang	3	0.7
	Acroperus harpae (Baird,1835)	Acrohar	53	12.3
	Alona affinis (Leydig, 1860)	Aloaff	175	40.5
	Alona costata G.O.Sars, 1862	Alocos	3	0.7
	Alona elegans Kurz 1875	Aloele	4	0.9
	Alona guttata G.O.Sars, 1862	Alogut	20	4.6
	Alona quadrangularis (O.F.Müller, 1785)	Aloqua	61	14.1
	Alonella excisa G.O.Sars, 1862	Aloexc	73	16.9
	Alonella exigua Kurz, 1874	Aloexi	3	0.7
	Alonella nana (Baird, 1843)	Alonan	9	2.1
	Camptocercus rectirostris Schoedler, 1862	Camrec	1	0.2
	Flavalona costata (G.O.Sars, 1862)	Flacos	3	0.7
	Graptoleberis testudinaria (Fischer, 1848)	Grates	5	1.2
	Pleuroxus aduncus (Jurine, 1820)	Pleadu	4	0.9
	Pleuroxus laevis G.O.Sars,1862	Plelae	2	0.5
	Pleuroxus striatus Schoedler,1863	Plestr	1	0.2
	Pleuroxus truncatus (O.F.Müller, 1785)	Pletru	3	0.7
Onychopoda				
Polyphemidae	*Polyphemus pediculus* (Linnaeus, 1761)	Polped	1	0.2
Cercopagididae	*Bythotrephes longimanus* Leydig, 1860	Bytlon	1	0.2
Haplopoda				
Leptodoridae	*Leptodora kindtii*	Lepkin	3	0.7

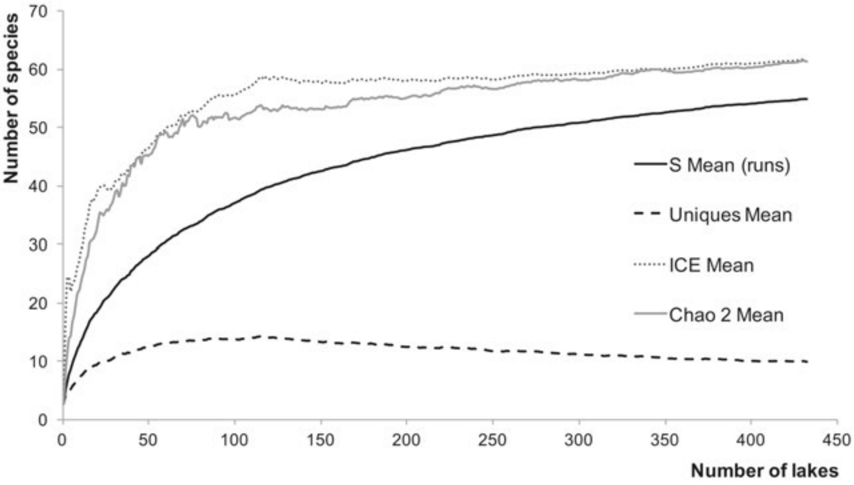

Figure 3. Species rarefaction curves of the cladoceran species richness in the Alpine lakes, variation of non-parametric estimators of species richness (ICE and Chao2) and of the number of uniques with increasing sampling size is illustrated. *Curvas de rarefacción de la riqueza de especies de cladóceros en lagos alpinos, variación de estimadores no paramétricos de la riqueza de especies (ICE y Chao2) y del número de valores únicos con incremento del tamaño de muestreo.*

0.25), Tmin ($r = 0.29$), Pm ($r = -0.23$) and, for a limited lake subset, water conductivity ($r = 0.25$, $n = 199$). Removing multicollinearity, in a multiple regression elevation (ALT) and solar radiation (DNI) better explained the variation of species richness ($p < 0.001$), together with spatial parameters dbMEMs 1 ($p < 0.01$) and 2 ($p < 0.001$). No significant relationships were found between local species richness and lake area, lake depth, and presence of predatory fish.

Overall beta-diversity of cladocerans in the Alpine region ($ß_{sor} = 0.999$) was almost completely caused by spatial turnover ($ß_{sim} = 0.988$, i.e., 99 %), with only a very small, negligible contribution from nestedness ($ß_{nes} = 0.006$).

The SOIUSA classification system divided the Italian Alps into 15 sections; 13 of them included sampled lakes. The degree to which differences in the composition of cladoceran assemblages (at 432 lakes throughout the Alps in 13 of the SOIUSA sections) matched this a priori regionalization was determined by analysis of similarities (ANOSIM) of Sorensen's similarity measures. ANOSIM analysis indicated that SOIUSA provided a statistically significant classification of cladoceran assemblages in the Alpine lakes (number of permutations: 9999, Global $R = 0.032$, $p < 0.02$). However, Global R was low, thus indicating that, although there was some overall difference in the cladoceran composition of the different regions, only a few of the individual zones could be considered to have compositions that distinguished them from all the other zones.

The total number of cladoceran species found in the Alpine geographical regions (regional gamma-diversity, Jackknife index) ranged from 13 to 44 species (25.9 ± 10.6, median 23). The geographical pattern of regional species richness is reported in Table 2. No longitudinal trend along the Alpine arc was observed; species-rich areas were located in the central (Dolomites and Rhaetian) Alps and Pre-Alps (Brescia and Garda), as well as in the Pennine Alps, whereas some species-poor areas were found in the western (Maritime), northern (Lepontine), and eastern (Carnic) Alps.

The overall beta-diversity pattern for Italian Alps, according to the $ß_{sim}$ -based cluster analysis, reflected two large clusters (Fig. 4a), roughly corresponding to (i) a western and southern Central Alps group and (ii) a northern and north-eastern group, while the central Pre-Alps and the two eastern areas (Carnic and Julian Alps) formed isolated clusters. The clustering classification was highly significant (ANOSIM, number

Table 2. List of the SOIUSA sections (SZ) of the Italian Alps where the sampled lakes were distributed; section code, area, altitudinal range (ALTr), number of lakes (Nlakes) and cladoceran species richness (SP, Jackknife estimates) are reported. *Lista de las secciones SOIUSA (SZ) de los Alpes italianos donde se distribuían los lagos muestreados; código de sección, área, rango altitudinal (ALTr), número de lagos (Nlakes) y riqueza de las especies de cladóceros (SP, estima con Jackknife).*

CODE	Section name	Area (km^2)	ALTr	Nlakes	SP
SZ.02	Maritime Alps	854.90	3286	26	12.9
SZ.04	Cottian Alps	3564.50	3556	37	22.8
SZ.07	Graian Alps	4077.06	4569	45	18.9
SZ.09	Pennine Alps	1619.32	4418	58	36.8
SZ.10	Lepontine Alps	1060.10	3345	29	17.9
SZ.15	Western Rhaetian Alps	2504.07	3800	15	13.8
SZ.16	Eastern Rhaetian Alps	1557.53	3496	18	27.5
SZ.28	Southern Rhaetian Alps	4823.41	3701	49	36.8
SZ.29	Bergamo Alps and Pre-Alps	3343.42	2855	47	27.9
SZ.30	Brescia and Garda Pre-Alps	2647.06	2188	9	40.2
SZ.31	Dolomites	6385.67	2475	83	43.9
SZ.33	Carnic Alps	4205.35	2638	9	14.3
SZ.34	Julian Alps	2996.84	2649	7	23.4

of permutations: 9999, number of permuted statistics greater than or equal to Global R: 0, Global R = 0.676, $p < 0.0001$). However, a careful examination of the possible pairwise tests showed that only the separation of the two main clusters in Fig. 4b was significant ($p < 0.001$), while the analysis failed in demonstrating the distinctiveness of the isolated clusters from the previous ones. A nMDS analysis (Fig. 4c, 2D stress = 0.14) illustrated better the relationship between the different sections and clusters.

Determinants of species distribution patterns

The distance-based redundancy analysis (dbRDA) explained only 15.4 % of the variation of the species-distribution matrix. The constrained variation explained by the first two axes of dbRDA was 54.5 %, indicating a high, significant correlation between spatial and environmental predictor variables and the species distribution similarity matrix. The environmental and spatial vectors superimposed on the plot as arrows (Fig. 5b) clearly showed that the major determinants of the first axis (explaining 30.2 % of constrained variation) were the spatial eigenvectors (dbMEMs 2-3), representing mainly longitudinal spatial gradients. The second axis (24.3 % of constrained variation) was an altitudinal gradient, defined by altitude and related morphometric (lake area, larger lakes being located at lower altitudes) and climatic (temperature, precipitation and evapotranspiration) factors, as well as by the medium-scale dbMEM 9, representing mainly a latitudinal spatial gradient. The geological settlement (linked to water chemistry) as well as solar radiation were weakly correlated with the dbRDA axes. The main subdivision of sampled lakes along the first axis was thus determined by the Moran's eigenvectors, which described in detail the spatial structure of the Alpine region. The graph clearly showed that, at a broad spatial scale, lakes were grouped into the two major SOIUSA clusters (Fig. 5a). However, this was not a merely longitudinal gradient, reflecting the results of the cluster analysis. Sections 31 (Dolomites), 10 (Lepontine Alps), 15 and 16 (Rhaetian Alps) (i.e. cluster 5) were projected on the right side of the graph (mainly determined by the spatial vector dbMEM2), while the western sections (2-7: Maritime, Cottian, Graian and Pennine Alps) together with sections 28 (Southern Rhaetian Alps) and 29 (Bergamo Alps and Pre-Alps), i.e. cluster 4, were projected on the left side (mainly defined by dbMEM3). Lakes of section 30 (Brescia and Garda Pre-Alps, cluster 1) were mapped in the lower part of the graph, i.e. were defined by lower elevation and higher temperatures and, to a lesser extent, higher surface area (SUP) and annual evapotranspiration (AET).

Being the amount of unexplained variance in the dbRDA quite high, the analysis was repeated on a subset of 199 lakes for which more parameters (depth, water conductivity, presence/absence of predatory fish, and natural/artificial origin) were available. The dbRDA analysis explained 19.8 % of total variation in the species distribution matrix. The first two axes (54.3 % of constrained variation, and 10.8 % of unconstrained variation) evidenced the role of depth and naturality in explaining an additional portion of species distribution variation, characterizing the first axis. The second axis reflected a climatic gradient of temperature, precipitation and AET, as in previous dbRDA analysis, while the presence of preda-

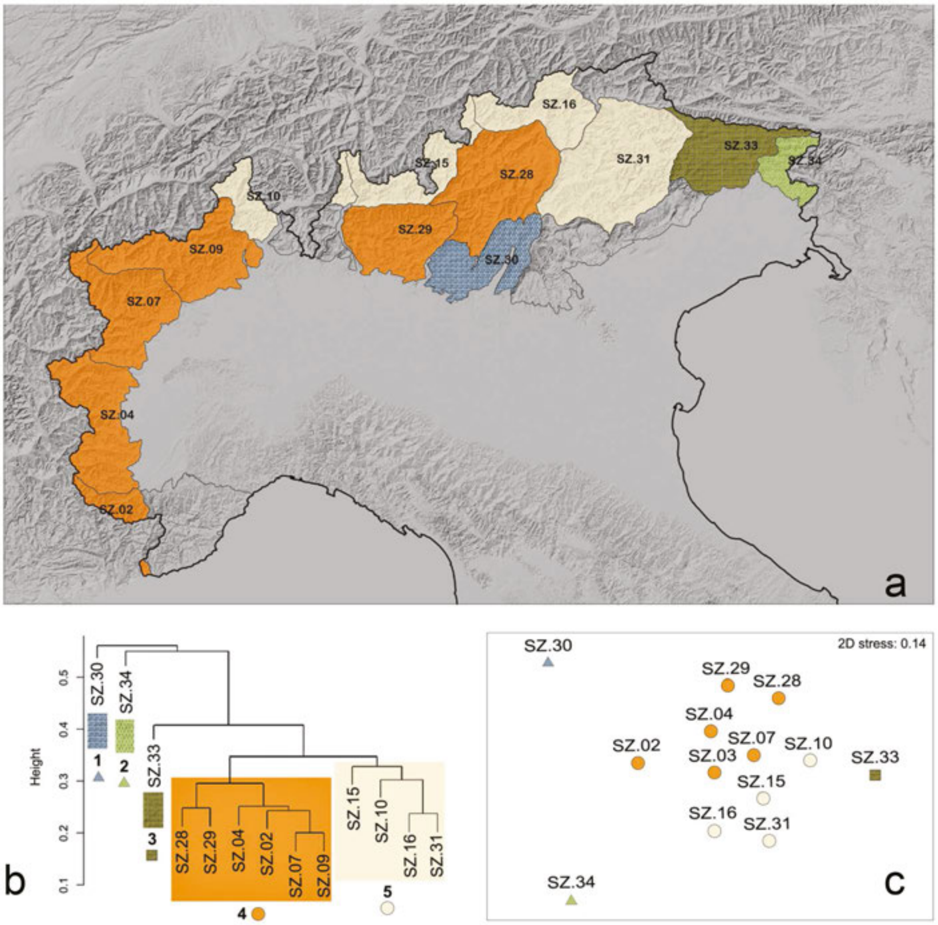

Figure 4. Results of the agglomerative hierarchical clustering analysis of SOIUSA sections based on the species turnover similarity matrix of cladoceran assemblages; codes as in Table 2. (a) Geographical distribution of the clusters in the Alpine ecoregion; (b) clustering dendrogram; (c) non-Metric Multidimensional Scaling (nMDS) ordination of the Alpine sections based on the same similarity matrix. *Resultados del análisis de agrupamiento jerárquico de las secciones SOIUSA. Basado en la matriz de similitud de recambio de especies de la comunidad de cladóceros. Los códigos son iguales que en la tabla 2. (a) Distribución geográfica de los grupos en la ecorregión alpina, (b) Dendrograma de agrupamiento; (c) Ordenación (nMDS) de las secciones de los Alpes basada en la misma matriz de similitud.*

tory fish, geological setting and conductivity was unimportant (the graph illustrating the relationships of variable with dbRDA axes is reported in the Supplementary information, available at http://www.limnetica.net/en/limnetica).

Although a canonical analysis based on SOIUSA sections has to be used with caution, as suggested by ANOSIM analysis, dbRDA was applied to Alpine areas as well to detect general, broad-scale trends, minimizing the influence of local scale parameters (Fig. 6a). dbRDA applied to SOIUSA sections explained 86.5 % of the species distribution pattern variation along the Alpine chain. The first two dbRDA axes explained 59 % of the constrained variation and 43 % of the total variation. The first axis was an altitudinal gradient, while the second axis was mainly defined by the dolomitic geological setting and the altitudinal heterogeneity. Species correlation with the first two axes was quite high (Fig. 6b). *Bosmina longispina*, *Eurycercus lamellatus*, *Simocephalus vetulus*, *Alona guttata* and *Diaphanosoma brachyurum*, among others, confirmed their link with low-elevation, warmer, and more productive areas. On the contrary, a group of species including *Macrothrix hirsuticor-*

nis, *Coronatella rectangula*, *Alona affinis*, *Acroperus angustatus* and *Daphnia middendorffiana* showed a preference for areas with higher elevation and lower temperature and productivity, while the presence of the widespread *Daphnia longispina* was consistent in the more heterogeneous areas, and quite distinct from the distribution of the similar *D. zschokkei*, *D. hyalina*, and *D. rosea*. Finally, a group of species including *Daphnia obtusa*, *Streblocerus serricaudatus*, and *Alonella excisa* was found mainly in the cluster of Dolomites, Lepontine and Rhaetian Alps.

DISCUSSION

To the best of our knowledge, the present study provides the first comprehensive assessment of cladocerans in the Italian Alpine lakes, using data compiled from literature sources as well as an extensive set of newly collected samples. In total, our research indicates that at least 55 cladoceran species inhabit the lakes of the Alpine ecoregion in Italy (i.e. over 40 % of the whole Italian cladoceran fauna, including 124 species: Margaritora, 1985, 2005 and unpublished checklist updates). Most species of cladocerans recorded in Alpine lakes are widely distributed across Europe as well as across continental Italy, occurring in several ecoregions (Margaritora, 2005); no one species is endemic to the Alpine ecoregion. The widespread occurrence of most species across Europe and the Alpine arc suggests that dispersal is probably not a factor limiting the occurrence of cladocerans in the studied lakes.

Only three species (*Daphnia longispina*, *Chydorus sphaericus* and *Alona affinis*) where widely distributed in the Alpine lakes. The amount of rare species collected in the area was quite high, with more than 80 % of the species present in less than 10 % of the lakes. Unfortunately, the logarithmic distribution fitted to the rank-frequency occurrence data do not offer any explanatory value for the process behind species distribution patterns. Applying a modified Yule's three-parameter model, Maruvka *et al.* (2011) acknowledged that many other non-biological objects, for example size of cities in a country, usage of words in human languages, or even the number of web sites with certain degree of links per year follow a hollow curve pattern. For this reason, any ecological explanation of log series distribution must be taken with caution.

The log series, introduced in ecology by Fischer *et al.* (1943), could have been generated by an insufficient and/or uneven sampling effort (Magurran, 2006), which prevented a complete survey of alpha-species diversity in lakes, artificially increasing the number of rare species. As

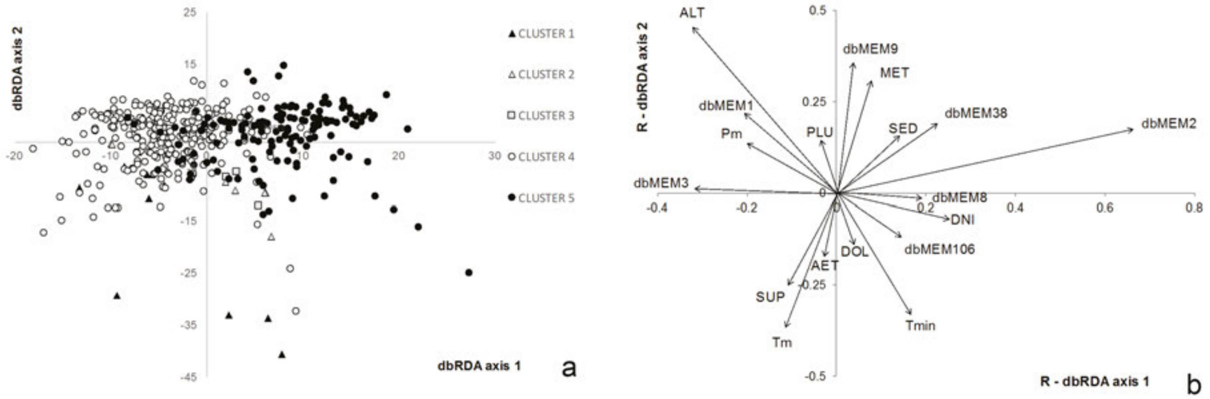

Figure 5. Results of distance-based redundancy analysis (dbRDA). (a) Plot of Alpine lakes on the plane defined by the first two canonical axes; markers correspond to the major clusters illustrated in Fig. 4. (b) Plot of the predictor variables on the plane defined by the first two canonical axes using their multiple correlation coefficients with the dbRDA axes; variable acronyms: see text. *Resultados del análisis de redundancia basado en las distancias (dbRDA). (a) Plot de los lagos alpinos en el plano definido por los dos primeros ejes canónicos. Los símbolos corresponden a los grupos principales ilustrados en la Fig. 4. (b) Variables predictoras; acrónimos de las variables: ver en el texto.*

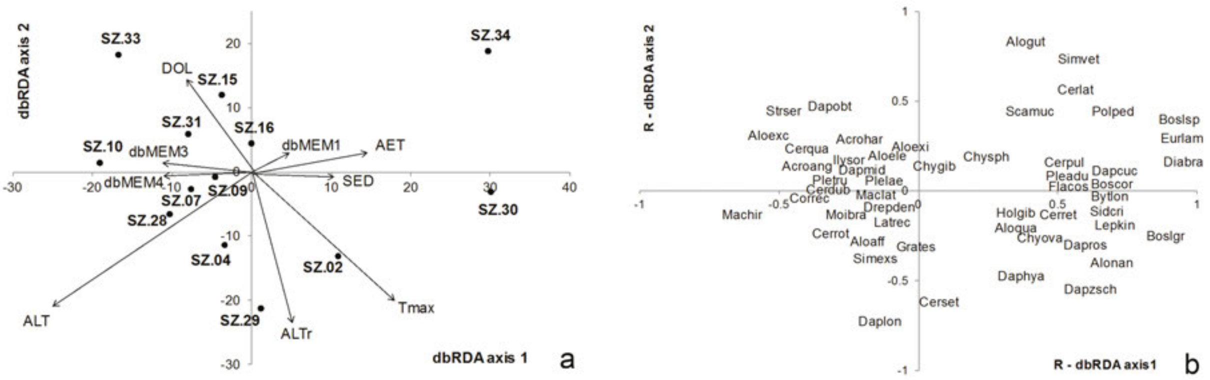

Figure 6. Results of distance-based redundancy analysis (dbRDA) applied to the SOIUSA sections. (a) Biplot of SOIUSA sections and predictor variables on the plane defined by the first two canonical axes; codes of sections as in Table 2; variable acronyms: see text. (b) Plot of cladoceran species on the plane defined by the first two canonical axes using their multiple correlation coefficients with the dbRDA axes; species acronyms as in Table 1. *Resultados del análisis de redundancia basado en las distancias (dbRDA) aplicado a las secciones SOIUSA. (a) Biplot de las secciones SOIUSA y las variables predictoras en el plano definido por los dos primeros ejes canónicos; los códigos son como los de la Tabla 2; los acrónimos de las variables: ver en el texto. (b) Biplot de las especies de cladóceros; los acrónimos de las especies igual que en la Tabla 1.*

with most large-scale surveys (Pinel-Alloul *et al.*, 2013), there are caveats in our study: our sampling did not cover the whole summer growing season and the surveys published in the literature used to compile the species distribution matrix extended through more than 70 years. However, species rarefaction curves indicated a decrease in the number of uniques after 120 sampled lakes, suggesting that sampling was quite efficient. Moreover, bottom-dwelling cladocerans (see Kotov, 2006 for their definition) are well represented both in the common (*Chydorus sphaericus*, *Alona afinis*) than in rare species (*Ilyocryptus sordidus*, *Lathonura rectirostris*, *Drepanothrix dentata*, *Pleuroxus truncates*) fractions, suggesting that sampling effort covered both open water (planktonic) and bottom-dwelling (benthic) habitat in the studied lakes.

Magurran (2006) stated that a log series best fit the species-abundance data in harsh environments where few factors determine the distribution patterns. This is the case of the Alpine ecoregion, and especially of high altitude lakes. Less extreme environmental conditions give rise to other kinds of distributions, as can be observed looking at the graph reported by Pinel-Alloul *et al.* (2013) for a set of 1665 Canadian lakes. However, most studies refer to the whole zooplankton crustacean community, including copepods, which have very different ecological life-cycle, so that Henriques-Silva *et al.* (2016) suggested to disentangle the study of the two groups in macroecological and biogeographical analyses. Cladocerans cope with winter conditions by going through a diapausing phase that resists freezing and drying (Havel & Shurin, 2004), providing long-term persistence in harsh environments. Moreover, cladocerans are mostly cyclic parthenogenetic organisms that produce diapausing eggs during the sexual phase; for this reason, they need at least two generations to produce resting eggs. Cladocerans dwelling in high-altitude lakes on the Alps experience cold temperatures; they have a shorter growing season and a slower metabolism than at lower altitudes. High-altitude conditions might thus exclude several species which are unable to complete their life cycle (Henriques-Silva *et al.*, 2016), explaining the decrease of species richness with altitude and decreasing temperature, observed in our analysis, and the high degree of rarity.

A further explanation of the excess of rare species may be found in cladoceran dispersal ways and colonization patterns (Henriques-Silva *et al.*, 2016). A post-glacial colonization of lakes may have given rise to a log series distribution: in analogy to species-abundance models (Magurran, 2006), if species arrived in an unsaturated area (like the Alpine chain after the Last Glacial Maximum) at irregular intervals of time, occupying the free fractions of remaining lakes, a log series distribution can be predicted. The monopolization hypothesis, proposed by De Meester *et al.* (2002), and lately extended to multiple species communities (De Meester *et al.*, 2016), may support our explanation. Following Henriques-Silva *et al.* (2016), we suppose that Alpine lakes were first randomly colonized after glaciers retreat by long-distance dispersal events (for example resting eggs carried by birds or winds: Incagnone *et al.*, 2014). Rapid population growth rates due to asexual reproduction enabled early colonizers to adapt quickly to the local environment (De Meester *et al.*, 2002), enhancing priority effects and precluding the establishment of later migrants through niche pre-emption (De Meester *et al.*, 2016). The monopolization hypothesis predicts the occupation only of free niches in remaining lakes by more recent colonizers, which originates the log series distribution of species frequencies.

Apart from statistical distributions, and despite the widespread occurrence of most species found in Alpine lakes, there were distinct differences in the relative frequency of cladocerans across Alpine regions, as described by cluster analysis and dbRDA, suggesting that factors other than dispersal may influence cladoceran distribution in Alpine lakes. We found that both spatial variables (which are mainly linked to dispersal) and climatic variables (mainly linked to the altitudinal gradient) explained the variation in cladoceran assemblage structure along the Alpine arc. Despite the highly significant correlation between spatial and environmental predictor variables and the species distribution similarity matrix, the total amount of variation explained considering lakes as sampling units in multivariate analysis was quite low, but comparable to other zooplankton surveys that used ordination techniques (Pinel-Alloul *et al.*, 2013; Sweetman *et al.*, 2010; Anas *et al.*, 2014). Leibold *et al.* (2010) demonstrated that cladocerans respond more to local-scale environmental factors in lakes than to regional-scale factors. Moreover, Anas *et al.* (2014) found that assemblage structure of crustacean zooplankton in northwest Saskatchewan lakes (Canada) were largely regulated by local environmental factors, even if the total variance explained, accounting also for local factors (including water chemistry and fish predation), did not exceed 24 %. We added some local explanatory variables (depth, water conductivity, presence/absence of predatory fish, and natural/artificial origin of the basins) using a subset of 199 lakes, increasing the explained variance approximately from 15 to 20 %, in agreement with Anas *et al.* (2014) results. The unexplained variation seems unavoidable and can be simply attributed to noisiness of species data and unmeasured local biotic factors; for this reason, its influence on the main results of our macroecological analysis seems negligible.

Clustering lakes within geographical macro-areas (like the SOIUSA sections used in our study), as usual in large-scale analyses (Pinel-Alloul *et al.*, 2013; Henriques-Silva *et al.*, 2016; Marrone *et al.*, 2017) allowed us to focus on broad-scale macroecological patterns, avoiding the effect of local scale variables. dbRDA applied to SOIUSA sections explained most (86.5 %) of the total variation of species distribution pattern. Ecological factors linked to the altitudinal gradient like actual evapotranspiration (i.e. a surrogate of productivity in freshwaters, see Eme *et al.*, 2015 and Stoch *et al.*, 2016), temperature and precipitation, together with the geological setting (influencing water chemistry) and altitudinal heterogeneity were the major ecological determinants of the large-scale cladoceran distribution in the SOIUSA sections across the Alpine chain. The role of solar radiation alone as a major determinant of regional species richness (Pinel-Alloul *et al.*, 2013) was not supported by our multivariate analysis conducted at the scale of the Alpine ecoregion. These results support the prediction of Henriques-Silva *et al.* (2016) regarding environmental filtering and a strong relationship to climatic factors for cladocerans. However, beta-diversity partitioning analysis performed by Henriques-Silva *et al.* (2016) showed that 83 % of the variation in cladoceran beta diversity ($ß_{sor}$) was due to pure spatial turnover and 17 % to nestedness, suggesting that cladoceran beta diversity in Canadian lakes was partially driven by species loss. Our study did not support this hypothesis, being nestedness negligible in Alpine cladoceran assemblages. The habitat heterogeneity of the Alpine chain, rich of barriers compared to the Canadian area studied by Henriques-Silva *et al.* (2016), as well as the difference of scale (our study concentrated on a single ecoregion) may be plausible explanations of this discrepancy, suggesting that species turnover alone explains the variation in species composition of lake assemblages along the Alpine chain. This result prevents a generalization of the hypothesis of the role of nestedness in shaping beta-diversity patterns in zooplankton along multiple spatial scales.

CONCLUSION

Our broad-scale survey of cladocerans in Alpine lakes gave additional support to the hypothesis put forward by Pinel-Alloul (1995), Pinel-Alloul *et al.* (2013) and Shurin *et al.* (2007) which stated that abiotic environmental gradients will be the most important drivers of spatial variation in zooplanktonic community structure at large scale. However, given the high dispersal ability of cladoceran species, their peculiar life strategy including the production of diapausing eggs, and their compliance to the monopolization hypothesis (De Meester *et al.*, 2002), allowed us to formulate a novel hypothesis on the origin and variation of cladoceran assemblages along the Alpine arc accounting for dispersal ability, life-cycle characteristics and environmental filtering. Our data suggest that this is a likely explanation of the excess of rarity in these environments, and of the observed log series distribution of species frequencies in the area. Moreover, the strong relationship between local cladoceran species richness and the altitudinal gradient (including all the ecological parameters that covary with altitude), evidenced by our analysis, reinforces the role of the peculiar cladoceran life cycle based on cyclic parthenogenesis in shaping species distribution and species richness patterns.

Although our results partly agree with the statements by Leibold *et al.* (2010), Anas *et al.* (2014), and Henriques-Silva *et al.* (2016) that cladocerans respond also to local-scale environmental factors in lakes, a macroecological approach revealed a large-scale distribution pattern which can be disentangled from local variables effects. Hypotheses related to the the importance of nestedness in shaping beta-diversity patterns (Henriques-Silva *et al.*, 2016) as well as the role of solar radiation as a major determinant of regional species richness (Pinel-Alloul *et al.*, 2013) were not confirmed at the scale of our extensive survey on Alpine lakes, emphasizing the presence of scale-dependent spatial and environmental determinants of cladoceran distribution patterns.

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How do zooplankton respond to coastal wetland restoration? the case of newly created salt marsh lagoons in La Pletera (NE Catalonia)

Santiago Cabrera*, Jordi Compte, Stéphanie Gascón, Dani Boix, David Cunillera-Montcusí, Lucas Barrero and Xavier D. Quintana

GRECO, Institute of Aquatic Ecology, University of Girona, 17003 Girona, Spain.

* Corresponding author: jscg88@gmail.com

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ABSTRACT

How do zooplankton respond to coastal wetland restoration? The case of newly created salt marsh lagoons in La Pletera (NE Catalonia)

We analysed the changes in the zooplankton composition in several new and old lagoons in La Pletera, a confined Mediterranean coastal salt marsh located in the Baix Ter wetlands (NE Iberian Peninsula) that was subjected to a restoration project (Life Pletera). We sampled the zooplankton from three new lagoons immediately after their creation (1 and 2 months after), and we compared the zooplankton with those of three existing lagoons, two natural lagoons and one lagoon created fourteen years ago in a previous restoration project. The sampling included seven months before the first hydrological connection to the surface waters, when flooding during a sea storm connected the new lagoons with the existing lagoons for the first time, and seven months after. The results showed that the nutrient and organic matter concentrations were lower in the new lagoons than in the old lagoons, suggesting accumulation processes in these ecosystems, which were probably related to the long periods of confinement that typically follow flooding events in this type of lagoons. However, we did not find significant differences in the zooplankton community between the old and new lagoons. The seasonal variation in the zooplankton communities was strongly affected by the seasonal hydrological flooding – confinement pattern, which did not differ between the old and new lagoons. Calanoid copepods, mainly *Eurytemora velox*, dominated during winter, while the rotifer *Brachionus plicatilis* dominated in summer, coinciding with the highest degree of confinement. The environmental variables and the hydric connection explained 31 % of the zooplankton variability. The high connectivity of the system once it flooded aided dispersal, the hatching of the egg banks existing in the restored lagoons and the fast response of the zooplankton species to changes in the environmental conditions (although with some degree of delay) might explain the lack of significant differences between the zooplankton composition in the old and new lagoons.

Key words: zooplankton, coastal lagoons, flooding confinement, restoration, high dispersal, egg banks

RESUMEN

¿Cómo responde el zooplancton a la restauración de un humedal costero? El caso de las lagunas recientemente creadas en la marisma de La Pletera (NE Cataluña)

Se analizaron los cambios en la composición del zooplancton en algunas lagunas de nueva creación y ya existentes de La Pletera, una marisma confinada en la costa del Mediterráneo, localizada en los humedales del Baix Ter (NE Península Ibérica) y sujeta a un proyecto de restauración Life (Life Pletera). Se muestreó el zooplancton de tres nuevas lagunas inmediatamente después de su creación (1 o 2 meses después) y se comparó con el de tres ya existentes; dos lagunas naturales y una creada catorce años antes en un proyecto de restauración previo. Los muestreos incluyeron siete meses antes de la primera conexión hídrica superficial, cuando el proceso de inundación durante los temporales de mar conectó por primera vez las nuevas lagunas con las ya existentes, y los siete meses posteriores. Los resultados mostraron que la concentración de nutrientes y materia orgánica fue más baja en las nuevas lagunas que en las viejas, sugiriendo un proceso de acumulación en estos ecosistemas, probablemente relacionada con los largos periodos de confinamiento que normalmente siguen a los eventos de inundación en este tipo de lagunas. Por otro lado, no se encontró diferencias significativas en la comunidad zooplanctónica *entre lagunas viejas y nuevas. El patrón temporal del zooplancton estuvo fuertemente afectado por el patrón temporal hidrológico de inundación-confinamiento, el cual no difiere entre lagunas viejas y nuevas. Los copépodos calanoides, principalmente* Eurytemora velox*, dominaron durante el invierno, mientras que el rotífero* Brachionus plicatilis *dominó en verano coincidiendo con el mayor grado de confinamiento. Las variables ambientales y la conexión hídrica explican un 31 % de la variabilidad del zooplancton. La elevada facilidad de dispersión entre lagunas ubicadas muy cerca y fácilmente conectadas durante los eventos de inundación, la eclosión del banco de huevos existente en las lagunas restauradas y la respuesta rápida de las especies zooplanctónicas a los cambios en las condiciones ambientales (aunque con algún grado de retraso), podría explicar la falta de diferencias significativas entre la composición del zooplancton en las lagunas viejas y nuevas.*

Palabras clave: *zooplancton, lagunas costeras, inundación-confinamiento, restauración, dispersión alta, banco de huevos*

INTRODUCTION

Coastal lagoons are ecosystems that suffer different kinds of anthropogenic threats (e.g., Miracle *et al.*, 1984; Carrillo *et al.*, 1987; Comín *et al.*, 1987; Vicente & Miracle, 1992; Pérez-Ruzafa & Marcos, 2005; Romo *et al.*, 2005). They occupy approximately 13 % of the coastal areas in the world (Kjerfve, 1994) and are surrounded by strongly populated areas since approximately 40 % of human populations live within 100 kilometres of a coast (Small & Nicholls, 2003; Neumann *et al.*, 2015). Coastal lagoons have an important ecological role as habitats for numerous species, highly productive areas and ecosystem services providers of important value to societies (Anthony *et al.*, 2009; Newton *et al.*, 2018). For this reason, some approaches try to overcome the challenge of integrating the management of coastal lagoons in the framework of global change while taking into account their vulnerabilities and potentialities (Lillebø *et al.*, 2015; Vallejo, 1982; Anthony *et al.*, 2009). In this context, the European Union considers coastal lagoons as a priority habitat type in its Habitats Directive (Directive 92/43/EEC) and includes several coastal lagoons in the Natura 2000 Network. Both directives seek to conserve biodiversity and habitats in natural ecosystems, with the main goal of restoring the ecological functionality of these systems and preserving their species richness (CCE, 1992; General Direction of Environment European Commission, 2017). Regarding the Mediterranean coastal area, urban and tourism development during the last decades of the 20th century have caused the disappearance or degradation of most of their coastal aquatic ecosystems. Thus, the restoration and recovery of the ecosystem services of these habitats are priorities in EU habitat management, which is why several Mediterranean coastal lagoons have benefited from financial assistance for restoration purposes, such as the Life Nature projects (Quintana *et al.*, 2018).

Frequently, restoration projects mostly focus on eutrophication control or on keeping a permanent clear water phase to improve water quality (Scheffer *et al.*, 1993). The restoration of an aquatic habitat can be a difficult process since the main ecological restoration goal to enable the functioning of all ecological processes in the restored system is not always fulfilled (Hobbs & Harris, 2001). Possible causes of unsuccessful restoration may be a lack of understanding about the integrated ecological functioning of the ecosystem being restored or difficulties in dispersion and colonization by organisms. Evaluating the success of wetland restoration is often based on comparisons without available reference sites, previous studies or paleolimnological studies (Drake & Naiman, 2000; Antón-Pardo *et al.*, 2013). This is often why the basic restoration of ecological functions or just the recovery of an aquatic system are not achieved. Some studies have suggested that structural and functional restoration could be achieved in the medium term (decades), but some characteristics such as plant assemblages or biogeochemical functioning could take longer amounts of time (centuries) or stabilize in an alternative state to the natural or reference conditions (Moreno-Mateos *et al.*, 2012 and 2015; Murcia *et al.*, 2014). Although the management of restored habitats requires looking forward for this kind of long-term response to recovery, the need to wait for decades to assess the success (or failure) of a restoration seems ineffective. Thus, faster indicators that might reveal expected (or unexpected) behaviours of restored habitats immediately after restoration are needed.

Zooplankton assemblages respond rapidly to variations in environmental factors such as those caused by hydrological instability in Mediterranean coastal ecosystems (Quintana *et al.*, 1998a, 1998b; Brucet *et al.*, 2005; Badosa *et al.*, 2006; Olmo *et al.*, 2016). However, other factors or processes such as dispersal ability or priority effects also affect the colonization dynamics and, consequently, the zooplankton composition of restored wetlands (Bilton *et al.*, 2001; Cohen & Shurin, 2003; Ortells *et al.*, 2012; Antón-Pardo *et al.*, 2016). These assemblages are dominated by passive dispersers that are characterized by a high dispersal ability by wind or bird transport (but see Incagnone *et al.*, 2015); thus, they rapidly colonize new water bodies (Louette & De Meester, 2005; Frisch *et al.*, 2006; Olmo *et al.*, 2012; Audet *et al.*, 2013). In addition, egg banks can remain viable for decades or longer, but under the right environmental conditions, diapausing eggs can develop rapidly and conform to the zooplankton community together with new settlers (Brendonck & De Meester, 2003). Therefore, the analysis of zooplankton composition could be useful to observe the effects of restoration in the short term, and dispersal along with egg bank establishment may be an effective method for species colonization in the restoration and recovery of ancient existing aquatic habitats.

We analysed the zooplankton composition in a recently restored Mediterranean coastal ecosystem, La Pletera, where new lagoons were created. La Pletera is a salt marsh affected by incomplete buildings intended for a residential estate in the late 1980s. A Life Nature project (Life Pletera, LIFE 13 NAT/ES/001001) has been recently developed with the aim of recovering the ecological functioning of the salt marsh by dismantling the unused urban features (breakwaters, promenade, accesses, and piles of debris) built during the construction process in the 1980s. Our aim was to compare the zooplankton community composition between the old existing lagoons and the newly created lagoons. Given the rapid dispersion of zooplankton communities and the possibility of a remaining egg bank, we hypothesize a similar zooplankton composition between the old and new lagoons.

METHODS

Study area

The study area is located in La Pletera, a Salicornia-dominated Mediterranean salt marsh area located in the Baix Ter Wetlands in the northeastern region of the Iberian Peninsula (Fig. 1). It is located between the urban centre of l'Estartit (Torroella de Montgrí, Girona) and the mouth of the River Ter. The site is one of the few marsh areas left on the Catalan coast, located immediately behind a foredune. This area presents a typical Mediterranean hydrological regime, and it is affected mainly by the presence of the sea. Water intrusions in the lagoons or flooding periods come from sudden sea storms, intense rainfall and groundwater flows. On the other hand, there are other long periods or confinement periods without surface water inputs, especially in summer and winter when the lagoons can even become dry (López-Flores *et al.*, 2006; Badosa *et al.*, 2006; Menció *et al.*, 2017).

In 1987, partial urbanization modified the landscape of the zone, leading to the disappearance of some water bodies and the loss of the hydric connection (HC) among the remaining water bodies (Badosa *et al.*, 2006; Quintana *et al.*, 2009). Later, due to changes in urban classification promoted by the Council of Torroella de Montgrí, La Pletera was designated as a natural area and protected from building. Currently, the whole marsh of La Pletera is a natural park and is included in the Nature 2000 network (Quintana *et al.*, 2009 and 2018). In the framework of a LIFE Restoration project (LIFE99NAT/E/006386), some new lagoons were built in 2002 that mainly sought to improve the populations of Iberian toothcarp (*Aphanius iberus*), an endemic fish of the Iberian Peninsula that is in danger of extinction (Doadrio *et al.*, 2011), by means of the creation of new lagoons that would increase the number of habitat refuges for the endangered fish. These new lagoons joined the existing lagoons: Bassa del Pi and Bassa del Fra Ramon. In 2014, a second LIFE project began in the zone, project "LIFE-Pletera" (LIFE13NAT/ES/001001), with the objective, among others, of recovering the ecological functioning of the marshes (www.lifepletera.com). The infrastructures built during an old and unfinished urban process, such as breakwaters, promenades, accesses and accu-

Figure 1. Map of the study site with the location of the six lagoons studied within La Pletera salt marshes. *Mapa del área de estudio con la ubicación de las seis lagunas estudiadas dentro de la marisma de La Pletera.*

mulated debris, were removed and substituted by a set of new coastal water bodies. Limnological monitoring was carried out in the existing lagoons and in the new lagoons when they were created with the aim of comparing the different physical, chemical, hydrological and biological components.

Field sampling and laboratory analyses

Six lagoons were sampled: three were designated as "old" lagoons: Bassa del Pi (BPI), Fra Ramon (FRA), which are both natural lagoons, and Pletera Nova (G02), which was created in 2002. The other three were new lagoons that were created in 2016 1 and 2 months before the sampling started; these lagoons were dredged until reaching an elevation below sea level and were naturally filled with infiltration from the subsurface water from the saltmarshes: L04, L01 and M03 (Fig. 1). For details on the water circulation, see Menció *et al.* (2017).

Sampling was conducted monthly, covering an annual cycle starting in April 2016 through May 2017. The sampling covered a dry period (summer 2016), when the water level was at its minimum, and a flooding period (between autumn 2016 and spring 2017). During sea storm events, when the water level is highest, all water masses become connected, allowing displacement through the whole marsh. The hydric connections among the entire marsh (between the old and new lagoons) occurs when the water level increases to 1 metre above sea level (Pascual & Martinoy, 2017). Thus, at this water level, dispersal via the water by organisms among the lagoons is feasible. After the creation of the new lagoons in April 2016, the water level in the marsh achieved a level of 1 m a.s.l. for the first time in October 2016.

A few days later, the water level decreased, and the different lagoons remained isolated again (Pascual & Martinoy, 2017). Thus, we can divide our study time into two periods: before and after hydric connection. In terms of organisms' dispersal ability, these two periods differ in the fact that during the first period, any organism propagule

Table 1. Mean values and standard deviation (in brackets) of the environmental variables measured during the study period in the different lagoons aDO, deviation from 100% oxygen saturation. *Valores medios y desviación estándar (en paréntesis) de las variables ambientales medidas durante el período de estudio en las diferentes lagunas aDO, desviación del 100 % de saturación de oxígeno.*

Variable	Lagoons					
	Old			New		
	BPI	FRA	G02	L04	L01	M03
Conductivity (mS/cm)	74.8 (±54.7)	63.2 (±18.5)	44.9 (±18.6)	36.2 (±13.6)	28.4 (±8.9)	54.5 (±28.5)
pH	8.4 (±0.3)	8.5 (±0.2)	8.6 (±0.3)	8.7 (±0.3)	8.6 (±0.3)	8.8 (±0.5)
Temperature (ºC)	16.7 (±6.5)	17.5 (±6.2)	16.7 (±6.4)	16.7 (±6.2)	16.8 (±6.2)	17.7 (±6.8)
aDO	40.8 (±30.5)	48.1 (±35.2)	39.4 (±51.7)	9.3 (±10.3)	14.9 (±21.9)	30.2 (±50.1)
NH_4^+ (mg/L)	1.9 (±3.8)	0.3 (±0.3)	0.3 (±0.5)	0.2 (±0.4)	0.2 (±0.3)	0.1 (±0.1)
NO_2^- (mg/L)	0.005 (±0.005)	0.004 (±0.001)	0.004 (±0.001)	0.006 (±0.009)	0.004 (±0.0)	0.004 (±0.0)
NO_3^- (mg/L)	0.05 (±0.1)	0.02 (±0.0)	0.02 (±0.0)	0.03 (±0.006)	0.02 (±0.0)	0.02 (±0.0)
PO_4^{-3} (mg/L)	0.1 (±0.1)	0.06 (±0.05)	0.03 (±0.04)	0.009 (±0.01)	0.02 (±0.03)	0.01 (±0.01)
TP (mg P/L)	0.3 (±0.3)	0.3 (±0.2)	0.2 (±0.2)	0.1 (±0.03)	0.1 (±0.03)	0.2 (±0.1)
TOC (mg C/L)	103.2 (±64.3)	40.1 (±18.3)	37.0 (±18.8)	30.3 (±11.8)	39.1 (±22.2)	35.2 (±12.6)
TIC (mg C/L)	87.6 (±24.6)	51.2 (±8.9)	58.8 (±14.5)	57.5 (±13.1)	64.1 (±24.2)	57.0 (±19.8)
Water level (cm a.s.l.)	35.2 (±30.9)	29.9 (±23.4)	33.1 (±20.6)	34.7 (±21.1)	35.6 (±20.7)	38.5 (±35.6)

could not reach the new lagoons from the old lagoons via the water.

Water samples were taken to integrate, as much as possible, the entire lagoon. The samples were taken on the surface or up to a maximum depth of 50 cm. The temperature, electrical conductivity (EC25), pH and dissolved oxygen were taken in situ using a Hach HQ30d portable multiparameter probe. The water level (WL) was measured as the height in cm above or below the average sea level by means of levels installed in the lagoons. The average sea level was obtained from the Meteorological Station of L'Estartit and refers to the average sea level from 1990 - 1995 (Pascual & Martinoy, 2017). A total of 125 ml of unfiltered sample was collected to analyse the total nutrients: total nitrogen (TN), total phosphorus (TP), total organic carbon (TOC) and total inorganic carbon (TIC). The water filtered by Whatman GFF filters was collected to analyse the dissolved nutrients: ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-), soluble reactive phosphate (PO_4^{-3}), dissolved organic carbon (DOC) and dissolved inorganic carbon (DIC). The concentration of organic nitrogen and phosphorus were obtained by subtracting the inorganic forms from the total samples were frozen until analysis. These analyses were carried out following standard methods (APHA, 1992).

The zooplankton samples were obtained after in situ filtration of 5 L of water through a 50 µm net fixed in 4 % formalin. To identify, count and measure the zooplankton taxa, an inverted microscope was used. All the calculations, plots and statistical analyses in this work were performed with these 50 µm samples. The organisms in these samples were identified at the lowest taxonomical level possible without specimen manipulation. Thus, most of the taxa were not distinguished at the species level in the statistical analyses. The copepods were distinguished as calanoids, cyclopoids and harpacticoids as well as by their stages (nauplii, copepodites and adults). For each taxon, 25 individuals were measured to estimate the organisms' body size and biomas, measured in dry weight (DW). Crustacean biomass has been estimated from the allometric relationship between weight and body length, and using pre-established equations. The rotifer and ciliate biomass was calculated by converting the volume into a dry weight (Ruttner-Kolisko, 1977; Malley *et al.*, 1989; Putt & Stoecker, 1989). The zooplankton in these ecosystems are normally dominated by few species of copepod that represent most of the biomass present in the lagoons (Quintana *et al.*, 1998b; Brucet *et al.*, 2005). To corroborate this pattern and determine which copepod species were present, additional seasonal samples were collected. In these samples, high concentrations of adult copepods were captured, which allowed us to identify the copepods at the species level. This additional sample was collected using a 20 cm-diameter dipnet (mesh size: 250 µm) and 20 sweeps (a push half a metre long) in different habitats of the lagoon. In each sample, 24 specimens of each copepods group were determined at the species level.

Data analysis

To analyse the differences among lagoons in their water composition, a PCA analysis was performed. All the environmental variables except pH were log transformed. The oxygen concentration was determined by how far it was from 100 % saturation and the absolute [%] deviation from saturation was measured [aDO = abs|100-%0|], as proposed by Vollenweider *et al.* (1998), and this value was used in the PCA (aDO) instead of the rough oxygen concentration. The season, age of the lagoons (old or new) and the hydric connection (before and after) were added as categorical variables to determine the differences among them. To check the significance of the results, a between-group analysis coupled with the multivariate analysis (the dudi.pca function in R studio), and the calculation of the inertia for each zooplankton group (the dudi.coa function in R studio) was performed.

To determine differences in the zooplankton composition among the lagoons, non-parametric multi-dimensional scaling (NMDS) was performed using the biomass of the different identified zooplankton taxa. The adult and copepodite stages of the calanoids, cyclopoids or harpacticoids were grouped, differentiating them from the nauplii stages. The Bray-Curtis similar-

Table 2. List of zooplankton taxa found in La Pletera salt marshes and their mean densities, with the standard deviation (in brackets) for each lagoon (µg DW/L). *Brachionus quadridentatus*, Ciliophora and *Colurella* sp. were not included in the statistical analyses. The taxa codes are in brackets. *Listado de taxones de zooplancton encontrados en la marisma de La Pletera y sus densidades medias con la desviación estándar (entre paréntesis) para cada laguna (µg DW/L).* Brachionus quadridentatus*, Ciliophora y* Colurella *sp. no fueron incluidos en los análisis estadísticos. Entre paréntesis, código de los taxones.*

Taxa	**Lagoons**					
	Old			**New**		
	BPI	FRA	G02	L04	L01	M03
Copepoda						
Calanoida (adults and copepodites) (A_C_CAL)	1772.3 (±3482.0)	340.1 (±863.7)	5703.7 (±19445.6)	216.5 (±753.6)	288.8 (±814.7)	297.9 (±762.6)
Cyclopoida (adults and copepodites) (A_C_CYC)	0.5 (±1.2)	0.8 (±2.5)	0.5 (±1.7)	0.2 (±0.5)	0.4 (±0.8)	1.6 (±3.6)
Harpacticoida (adults and copepodites) (A_C_HAR)	0.2 (±0.9)	4.0 (±13.1)	6.4 (±17.9)	0.1 (±0.1)	0.2 (±0.6)	0.5 (±1.2)
Calanoida (nauplii) (N_CAL)	41.8 (±80.1)	302.0 (±983.5)	78.7 (±184.8)	76.1 (±280.9)	54.6 (±185.1)	55.7 (±191.4)
Cyclopoida (nauplii) (N_CYC)	1.0 (±1.7)	1.8 (±4.6)	3.1 (±6.4)	2.0 (±6.4)	0.7 (±0.8)	1.1 (±2.0)
Harpacticoida (nauplii) (N_HAR)	0.003 (±0.01)	0.03 (±0.08)	0.005 (±0.01)	0.005 (±0.01)	0.002 (±0.004)	-
Rotifera						
Asplanchna sp. (ASP_SP)	1.5 (±5.5)	-	-	-	-	-
Brachionus plicatilis (BRA_PLI)	4428.3 (±13963.2)	344.0 (±1233.7)	104.9 (±219.9)	26.9 (±92.4)	53.2 (±125.9)	566.9 (±2004.6)
Brachionus quadridentatus (BRA_QUA)	-	-	0.004 (±0.01)	-	-	-
Colurella sp. (CLR_SP)	-	-	0.0003 (±0.001)	-	-	-
Synchaeta sp. (SYN_SP)	0.2 (±0.6)	0.0002 (±0.0006)	1.0 (±3.6)	0.8 (±2.9)	1.8 (±6.8)	-
Testudinella clypeata (TES_CLY)	-	-	0.1 (±0.4)	-	0.0006 (±0.002)	-
Protozoa						
Ciliophora (CIL_SP)	-	0.006 (±0.02)	-	-	-	-

ity coefficient was calculated as the resemblance measure. The zooplankton data were transformed using the Hellinger transformation (Legendre & Gallagher, 2001). To detect possible associations between the patterns of species composition and the environmental variables, we used the 'envfit' function in the 'vegan' package in R, and statistical significance was evaluated by 999 random permutations. Finally, we also made a variance partitioning to determine the relative influence of the environmental variables (only including variables significantly related to zooplankton that were identified using a forward selection procedure), lagoon age and hydric connection on the zooplankton composition using zooplankton composition as a response matrix and the other variables as predictor matrices (Borcard *et al.*, 1992; Legendre & Legendre, 1998). We use the vegan package (Oksanen *et al.*, 2009) of the R language (R development

Core Team, 2008). All the calculations and statistical analyses were performed with R Studio 3.4.0 for Windows.

RESULTS

Environmental variables

The data measured in situ showed that there were no differences in water level between the old and new lagoons, with high variability and marked temporal patterns in each lagoon (Fig. 2a). The maximum increase started in October 2016, coinciding with the beginning of the flooding period, which was the first time the newly created lagoons connected with the existing lagoons (see methods). The conductivity was higher in the old lagoons until October 2016, when lagoons were connected (Fig. 2b). After that time, the conductivity values were similar in all the lagoons. The conductivity increased in the dry seasons, sometimes reaching or exceeding the seawater conductivity in the old lagoons, especially in Bassa Pi (BPI) and Fra Ramon (FRA), with maximum values of 193.5 mS/cm and 91.5 mS/cm, respectively. In the flooding period, the conductivity tended to decrease, with the lowest conductivity values reached in February (12.84 mS/cm in BPI and 18.82 mS/cm in L01). Table 1 summarizes the main environmental variables and their variability in the old (BPI, FRA and G02) and new lagoons (L04, L01 and M03).

The first two axes of the PCA analysis, which include the environmental variables, accounted for 48 % of the total variance (Fig. 3a). The first PCA axis (32 %) correlated negatively with water level and positively with conductivity, temperature and aDO. This means that this axis was related to the hydrological regime (flooding – confinement). The second axis (16 %) was related to the inorganic forms of N and C. It was positively correlated with NO_3^-, NO_2^- and DIC and negatively correlated with pH and particulate carbon (PIC and POC). The sample positions in the PCA plot differed (between-group analyses: $p < 0.001$) before and after the hydric connection (Fig. 3b). The samples taken before the hydric connection were more related to confinement and to higher organic nutrient content (higher PCA1 and PCA2 values), while the samples taken after the hydric connection were more related to flooding and to higher concentrations of inorganic nitrogen (lower PCA1 and PCA2 values). The sample positions in the PCA plot also differed (between-group analyses: $p < 0.001$) between the old and new

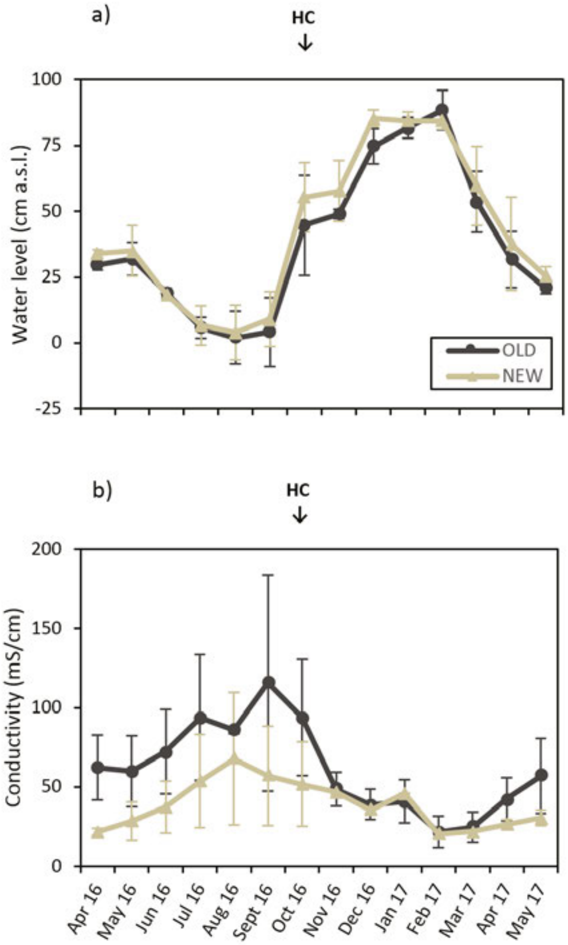

Figure 2. (a) Changes in the average water level (cm above or below sea level) of the studied lagoons (old and new) during the hydrological cycle 2016-2017. HC indicates the hydric connection for the first time. (b) Changes in conductivity (mS/cm) in the same lagoons (old and new) during the same period. *(a) Cambios en el promedio del nivel de agua (cm por encima o debajo del nivel del mar) de las lagunas de estudio (viejas y nuevas), durante el ciclo hidrológico 2016-2017. HC indica la conexión hídrica por primera vez. (b) Cambios en la conductividad (mS/cm) en las mismas lagunas (viejas y nuevas), durante el mismo período.*

lagoons, with higher PCA1 values in the old lagoons (Fig. 3c). The samples clustered by season showed significant differences, separating the samples from the dry periods (summer 2016) from those from the flooding season (autumn 2016 and winter 2016) ($p < 0.001$, in both cases). The springs (2016 and 2017) compared to winter also showed a significant difference ($p < 0.001$, in both cases). However, there was an interannual difference between spring 2016 and 2017 ($p < 0.05$) (Fig. 3d).

Zooplankton community structure and composition

Thirteen zooplankton taxa were identified in the samples (Table 2); three of these taxa were removed from the statistical analyses due to their low presence and abundance (those that appear in only one sample and with abundance below 1.0 µgDW/L): *Colurella* sp, *Ciliophora* and *Brachionus quadridentatus*. The copepods were dominated by *Eurytemora velox* in the calanoid

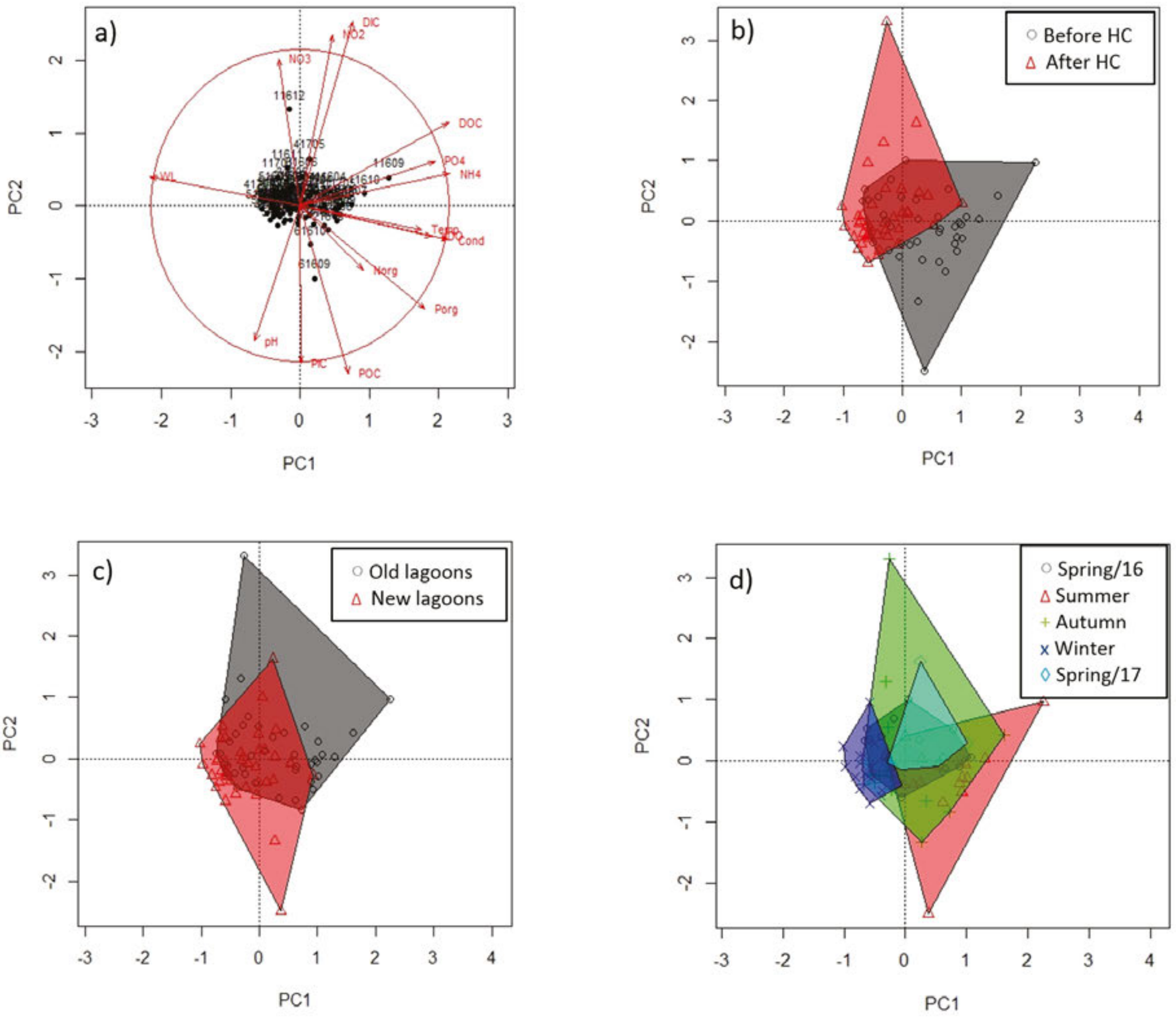

Figure 3. (a) PCA ordination diagram showing the distribution of the samples in relation to the environmental variables in the space represented by the first two axes. PCA polygons: (b) hydric connection [HC], (c) age and (d) season. *(a) Diagrama de ordenación PCA con la distribución de las muestras en relación con las variables ambientales representados en el espacio de los dos ejes principales. Polígonos PCA: (b) Conexión Hídrica [HC], (c) Edad y (d) Estación.*

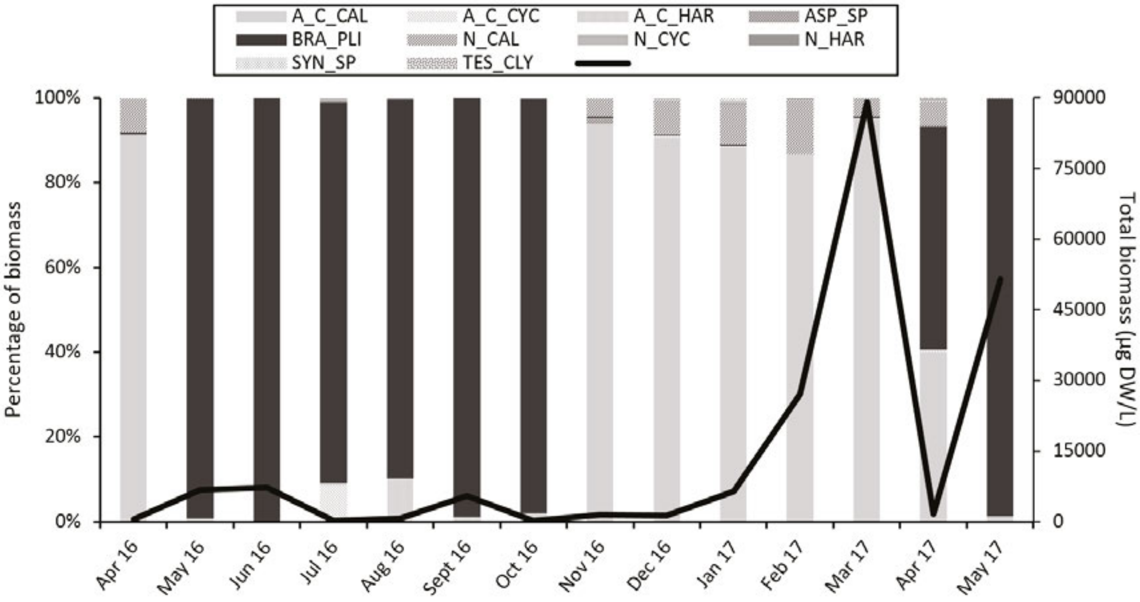

Figure 4. Relative abundance (in %) of the zooplankton species during the study period from 2016-2017 in the six lagoons. Total zooplankton abundance (µg DW/L) is shown by the solid line on the right axis of the plot. *Abundancia relativa (en %) de las especies de zooplancton durante el período de estudio 2016-2017 en las seis lagunas. La abundancia total del zooplancton (µg DW/L) se muestra en línea gruesa en el eje derecho del gráfico.*

adults (87 % of the individuals examined), *Halicyclops rotundipes* in the cyclopoids (86 %) and *Cletocamptus confluens* in the harpacticoids (more than 66 %). The most represented taxa were calanoid adults and copepodites, with the highest average density of 5700 µg DW/L corresponding to G02, and *Brachionus plicatilis*, with the highest average density of 4420 µg DW/L corresponding to BPI (Table 2). These two groups marked the temporal pattern in species composition. The calanoids dominated the community in the flooding period (up to 80 % of the total zooplankton biomass), while *B. plicatilis* dominated in the dry period (up to 85 % biomass) (Fig. 4). April 2017 was the only month when there was no clear dominance, but this time point divides the organisms' density by a similar percentage (approximately 45 %). In March 2017, the zooplankton biomass peaked, reaching a maximum of 89 200 µg DW/L, followed by a strong decrease and return to the average abundance values (3040 µg DW/L) (Fig. 4). The calanoid nauplii had a lower density than the previous two taxa, reaching a mean density of 330 µg DW/L in FRA. Their maximum densities coincided with the dominance of the calanoid adult stages (from November 2016 to April 2017) (Fig. 4).

The results showed differences among the lagoons in total zooplankton biomass. The old lagoons had a higher density, with a maximum value of 59 200 µg DW/L, while the new lagoons reached the density maximum value of 7300 µg DW/L (Fig. 5a). Lagoons BPI and G02 reached the highest densities (81 100 and 82 600 µg DW/L, respectively), but their compositions were different. In G02, calanoids dominated the community, reaching 96.7 %, while in BPI, these organisms represented 28.4% of the zooplankton community, with 70.9 % dominated by *B. plicatilis* (Fig. 5b). The other lagoons had a similar total biomass, although in order of density, FRA reached 13 900 µg DW/L with a similar proportion represented by calanoids (adults and copepodites), the rotifer *B. plicatilis* and the calanoid nauplii (Fig. 5). Within the new lagoons, M03 had a total zooplankton biomass of 12 000 µg DW/L, with a higher percentage of *B.*

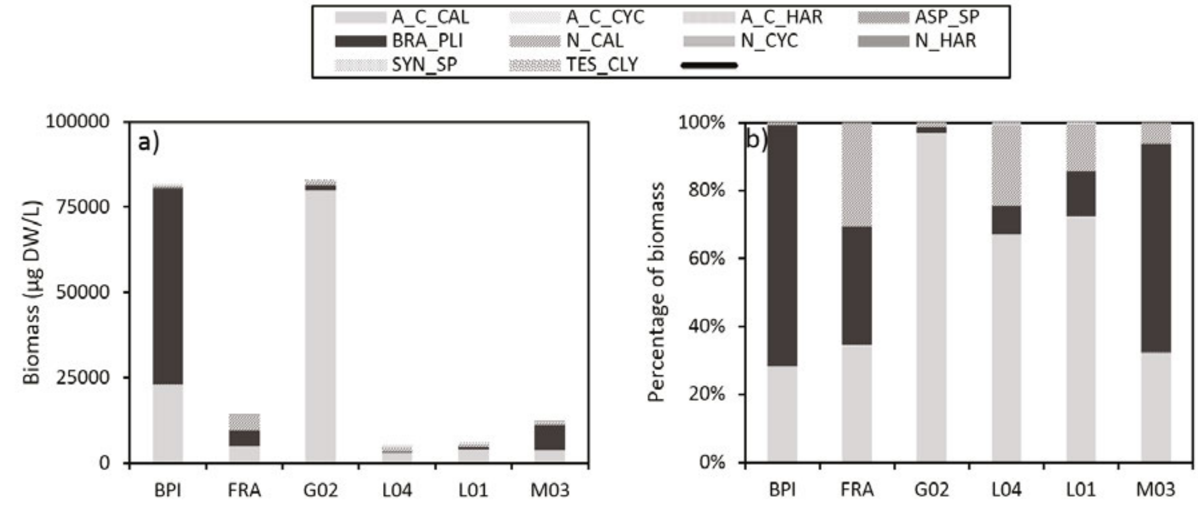

Figure 5. (a) Total biomass (µg DW/L) of the zooplankton composition in each lagoon [average of all months]. (b) Relative biomass percentage of the different zooplankton groups in each lagoon [average of all months]. *(a) Biomasa total (µg DW/L) del zooplancton en cada laguna [promedio de todos los meses]. (b) Porcentaje de biomasa relativo de los diferentes grupos zooplanctónicos en cada laguna [promedio de todos los meses].*

plicatilis (61.37 %) than calanoids (32.25 %). The other two new lagoons, L04 and L01, had similar total zooplankton densities (4520 and 5600 µg DW/L, respectively) and the same composition, with a dominance by calanoids (Fig. 5b).

The NMDS analysis results including the zooplankton taxa are listed in Table 2 and summarized in Fig. 6, showing a similar pattern to the one observed for the environmental variables (PCA results). The significant variables were the water level (in the lowest coordinates) ($p < 0.001$), which represented the flooding period dominated by the calanoids (all stages) and the rotifers *Testudinella* sp., *Asplanchna* sp. and *Synchaeta* sp. On the other hand, the samples with the highest coordinates were collected during the dry period when the conductivity ($p < 0.001$) and temperature ($p < 0.01$) were highest. Coinciding with the maximum values of aDO ($p < 0.01$), NH_4^+ ($p < 0.001$), SRP ($p < 0.01$) and the organic nutrients such as organic phosphorus ($p < 0.001$), organic nitrogen ($p < 0.05$) and DOC ($p < 0.001$), *B. plicatilis* dominated the community and had fewer cyclopoids and harpacticoids (adults and copepodites stages) (Fig. 6a).

Contrary to the results obtained in the PCA with the environmental variables, there were no significant differences in the zooplankton species composition. The sample positions in the NMDS results were not significantly different for the hydric connection (before and after) or the lagoon age (old and new lagoons) (Fig. 6b and 6c). Fig. 6b shows a strong overlap of the samples before and after the hydric connection, with the before-connection polygon surface including all the data of the after-connection polygon. The same situation occurred for the lagoon age, where the old lagoons overlapped the new lagoons (Fig. 6c). The seasons showed a certain degree of overlap among them, although the Monte Carlo test showed that there were some significant differences between winter and summer (flooding and dry period) ($p < 0.001$) (Fig. 6d). Summer and autumn also showed differences ($p < 0.01$), and winter presented differences with spring 2016 ($p < 0.001$) and spring 2017 ($p < 0.001$). Finally, the NMDS polygon results did not show a significant difference between the spring 2016 samples and spring 2017 samples, which differed from the result regarding the environmental variables (Fig. 5d).

The variance partitioning analysis showed that unique environmental variables explained 16 % of the variance in the zooplankton composition ($p < 0.001$). The environmental variables

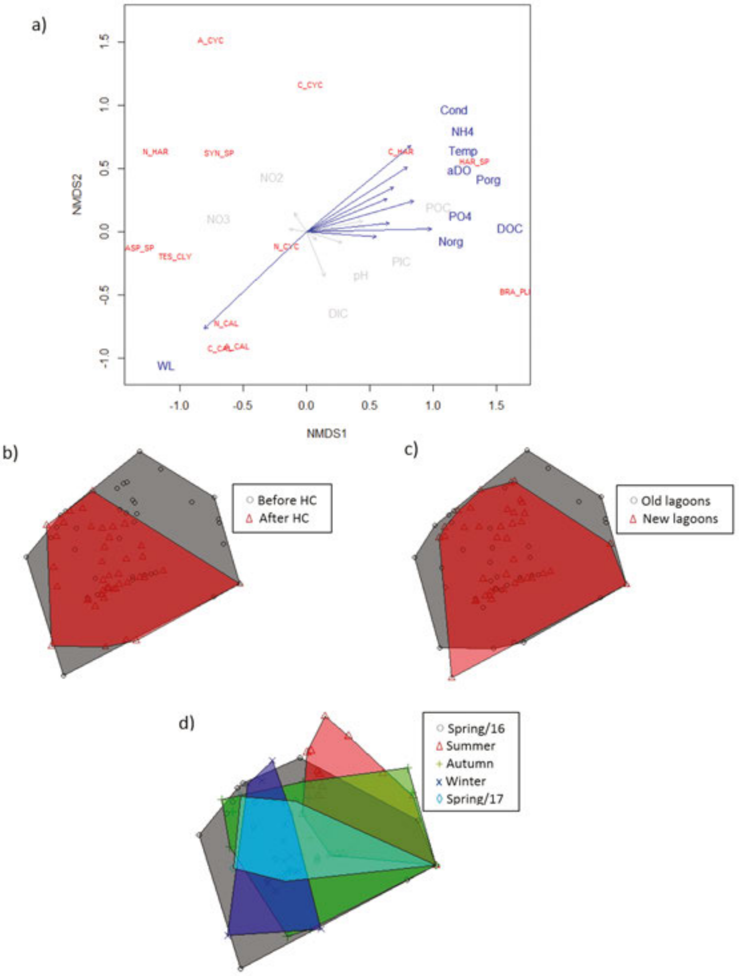

Figure 6. (a) NMDS plot, based on Bray-Curtis similarity, showing the distribution of samples and the zooplankton taxa (see taxa codes in Table 2) in relation to the environmental variables in the space. The polygons discriminate the samples by (b) hydric connection [HC], (c) age and (d) season. *(a) Gráfico NMDS, basado en los criterios de similitud de Bray-Curtis, mostrando la distribución de las muestras y los taxones de zooplancton (ver códigos de taxones en la Tabla 2) en relación con las variables ambientales. Los polígonos discriminan muestras por: (b) Conexión hídrica [HC], (c) Edad y (d) Temporada.*

sharing effects with the hydric connection explained 31%. The unique effects of lagoon age and hydric connection had no significant contribution to the variability in the zooplankton composition. Finally, 53 % of the variance remained unexplained.

DISCUSSION

The zooplankton composition in La Pletera is dominated by euryhaline species that are adapted to the high salinity fluctuations that characterize these ecosystems, which is similar to that found in other Mediterranean coastal environments with fluctuating environmental conditions (Cognetti & Maltagliati, 2000; Ramdani *et al.*, 2001; Antón-Pardo & Armengol, 2010). Species diversity is always low, with some single taxa dominating the zooplankton community (> 80 % dominance in biomass in at least 55 of the 82 samples analysed). This coincides with previous studies developed in these confined coastal marshes and in similar marshes (Quintana *et al.*, 1998b; Brucet *et al.*, 2005; Badosa *et al.*, 2006). We found higher differences among seasons, even in a single lagoon, than between the old and new lagoons. The seasonal variability in the zooplankton composition in La Pletera follows the flooding – confinement pattern that drives nutrient dynamics and species composition in this type of habitat (Brucet *et al.*, 2005): *Synchaeta* appears immediately after marine water intrusion; cyclopoid copepods appear in the middle season with a slight preference of the nauplii stages over the adult stages for flooding conditions; calanoid copepods dominate during stable winter-spring hydrological conditions; and *B. plicatilis* dominates during the dry period, with high confinement and frequent hypertrophic events due to the tolerance of this species to low oxygen concentration (Miracle *et al.*, 1987). Harpacticoid copepods have a brief dominance when the water level is low, but their resistance to severe confinement conditions is much lower than that of *B. plicatilis* (Moreira *et al.*, 1982). In our case, this pattern appears to be simpler than that observed by Quintana *et al.* (1998b) and Brucet *et al.* (2005). At least in part, this could be attributable to the sampling frequency, which was weekly in the cited references and monthly in this study.

The PCA results using the environmental variables showed some differences between the old and new lagoons. The total nutrients and organic matter concentrations were higher in the old lagoons, suggesting a progressive accumulation during successive flooding – confinement events over the years (Badosa *et al.*, 2006). However, we did not find significant differences between the old and new lagoons in the zooplankton NMDS results, suggesting a fast response of zooplankton after lagoon creation. Similar fast colonization has been reported in other studies (Badosa *et al.*, 2010; Antón-Pardo *et al.*, 2013). The environmental variables and hydric connection explained 47 % of the variance in the zooplankton composition. The remaining 53 % unexplained variance could be the consequence of several factors, such as hydrological parameters, "bottom-up" effects by other environmental variables that were not measured or "top-down" control by predators (Kelly *et al.*, 2002; López-Flores *et al.*, 2006; West *et al.*, 2009; Compte *et al.*, 2012).

Four different mechanisms may determine the zooplankton composition in newly created lagoons: 1) the dispersal ability and arrival of new colonizers from nearby water bodies, 2) the hatching of the egg bank present in the sediment of the new lagoons, 3) the physical and chemical composition of the new lagoons and 4) top-down control by predators. Zooplankton species such as other small organisms (i.e., small propagules) have been considered to have virtually no dispersal limitation according to the so-called 'cosmopolitan paradigm' or 'everything is everywhere' hypothesis (O'Malley 2007). This paradigm has been supported by (1) the rapid colonization of newly created or restored waterbodies by these organisms (e.g., Louette & De Meester, 2005; Olmo *et al.*, 2012; Audet *et al.*, 2013); (2) the high potential for passive long-range dispersal of organisms producing resting stages (Louette & De Meester, 2005); or (3) cysts being efficiently transported by physical or biological vectors (e.g., Bohonak & Whiteman, 1999; Vanschoenwinkel *et al.*, 2009). However, the generality of the cosmopolitan paradigm is under discussion (Incagnone *et al.*, 2015 and references therein), since: (1) spatial structures have been observed in plankton metacommunities even in ponds that were affected by similar environmental variables (Jenkins & Buikema, 1998); (2) high degrees of endemism have been reported for microcrustaceans (Marrone *et al.*, 2013); and (3) lower-than-expected genetic flows have been described for high-dispersal species (De Meester *et al.*, 2002). In La Pletera salt marshes, there is a short distance between the new lagoons and the existing ones, facilitating the colonization of the new lagoons. Antón-Pardo *et al.* (2016) mention that the short distance among the lagoons and the apparent absence of geographical barriers facilitate the occurrence of zooplankton dispersal vectors (e.g., waterbirds, wind or humans). Other studies performed on permanent and temporary lagoons showed a similar pattern to that found in the La Pletera salt marshes (Cottenie *et al.*, 2003; Audet *et al.*, 2013; Antón-Pardo *et al.*, 2016).

The lack of significant differences in zooplankton composition between the old and new lagoons before and after hydric connection suggests that zooplankton dispersion via hydrocora is not relevant in the early colonization of new lagoons. On the other hand, the hatching of the egg bank resting on the sediment filled with man-made debris during the last decades but removed during the restoration actions seems to be important. In fact, the new lagoons were built on what was a uniform marsh only a few decades ago. Thus, the new bottoms of the restored lagoons must be rich in resting eggs, which easily hatch after several decades of diapause (De Stasio, 1990; Alekseev & Starobogatov, 1996; Hairston & Cáceres, 1996). Egg banks are frequently dense in previously filled ponds, providing great opportunities for hatching immediately after restoration (Antón-Pardo & Armengol, 2010). Several studies support the importance of egg banks in facilitating colonization by zooplankton (Keller & Yan, 1998; Cohen & Shurin, 2003; Antón-Pardo & Armengol, 2010; Frisch *et al.*, 2012; Antón-Pardo *et al.*, 2013) as well as vegetation and invertebrates (Brouwer & Roelofs, 2001; Jenkins & Boulton, 2007; Ortells *et al.*, 2012; Antón-Pardo *et al.*, 2013) after pond restoration. In any case, due to our sample design, it is impossible to effectively distinguish between the dispersal and egg bank contributions to the zooplankton communities of the new lagoons. Nevertheless, both mechanisms likely act together to enhance community recovery after lagoon restoration.

We found differences between the new and old lagoons in their physical and chemical water compositions but not in their zooplankton compositions. However, some environmental variables, such as water level, conductivity and nutrient concentrations (especially organic nutrients), were related in the NMDS analysis of the zooplankton samples. This would suggest that the differences in the environmental variables are not large enough to cause a substantial modification in zooplankton species composition. Water level and salinity fluctuations are characteristic of aquatic ecosystems in Mediterranean climates (Alvarez-Cobelas *et al.*, 2005; Beklioglu *et al.*, 2007), and salinity has been described as an important driver determining community structure (Boix *et al.*, 2008) and species distribution at a regional scale (Gascón *et al.*, 2016) in Mediterranean wetlands. The strong variability in salinity and nutrient concentration is one of the main constraints that limit the colonization of these habitats to just a few euryhaline species adapted to this variability. Although strict marine or freshwater species may arrive at these lagoons during sea storms or flooding events, they rarely achieve stable populations if they cannot tolerate salinity fluctuations (Quintana *et al.*, 1998b; Brucet *et al.*, 2005). Regarding seasonal variations in species composition, once a zooplankton species tolerates the environmental filtering of salinity variability, its relative abundance follows the flooding – confinement pattern described for these confined coastal lagoons (Quintana *et al.*, 1998b). The zooplankton response to this pattern, however, arises with some degree of delay (Brucet *et al.*, 2005; Badosa *et al.*, 2007), which makes it difficult to establish the relationship between species composition and the physical and chemical variables. A dependence on the water turnover rate with different degrees of delay has also been observed in nutrient and chlorophyll-*a* concentrations (Quintana *et al.*, 1998a). Thus, environmental variability affects zooplankton species composition at two different scales. First, it limits the species presence to those that can overcome the constriction caused by strong environmental variability. Second, it determines species dominance by means of the flooding – confinement pattern to which zooplankton respond with a certain degree of delay. Since the new and old lagoons in La Pletera saltmarshes do not differ at these two scales, the zooplankton composition does not show significant differences between these types of lagoons.

Regarding top-down control, fish were absent in the new lagoons in the first months before the hydric connection, and the fish density was low in the new lagoons in the first months after connection (unpublished data). Thus, it seems that fish predation pressure has not had a strong effect on the zooplankton composition in these habitats since the composition was similar between the old and new lagoons even before the hydric connection. López-Flores *et al.* (2006) suggest the larger importance of bottom-up control in aquatic ecosystems strongly affected by environmental constraints. Badosa *et al.* (2007) found a smaller effect of fish predation on species composition in the same lagoons but a stronger effect on zooplankton size distribution. Moreover, fish predation pressure is also subject to seasonal variability (being higher in summer), as are the physical and chemical characteristics of water. Thus, it is likely that the effects of fish predation and water characteristics have certain covariations. Regarding macroinvertebrates, two potential predator species may achieve high densities in these lagoons: the amphipod *Gammarus aequicauda* and the Heteroptera *Sigara stagnalis*. The former appears in high densities during spring and summer, especially in the new lagoons, while the latter only peak occasionally in space and time (unpublished data). Although the main diet is not based on zooplankton in either species (Savage, 1989; Tachet *et al.*, 2000; Monakov, 2003), both taxa may predate zooplankton (Murillo & Recasens, 1986; MacNeil *et al.*, 1997; Kelly *et al.*, 2002; Compte *et al.*, 2012). Thus, a certain top-down control caused by invertebrate predation should not be discarded.

Restoration is important in terms of community succession and colonization processes, and substantial enhancement in species richness and the appearance of many new species after restoration have been reported (Antón-Pardo & Armengol, 2010; Antón-Pardo *et al.*, 2013). Our results show that zooplankton may be useful as a proxy for short-term restoration success due to their fast response and high dispersal ability. Of course, it does not necessarily mean that the whole ecosystem achieves maturity so quickly. Plants often colonize restored habitats more slowly, thus conditioning the recovery of the structural and functional attributes of aquatic ecosystems (Williams *et al.*, 2008; Ruhí *et al.*, 2016). Benthic macroinvertebrate colonization and succession dynamics after restoration have been described as fast and usually related to dispersal abilities (Ruhí *et al.*, 2009; Miguel-Chinchilla *et al.*, 2014), but they are affected by differences in habitat structure, such as those provided by plants (Cañedo-Argüelles *et al.*, 2011; Kim *et al.*, 2014). Thus, although wetlands are ecosystems with high turnover rates, the recovery of all of the functional and structural attributes after a restoration might require a long period of time (Moreno-Mateos *et al.*, 2012 and 2015). In summary, restoration effectiveness in coastal lagoons cannot be assessed without the use of several structural and functional indicators that usually respond in the long term. However, rapid response indicators such as zooplankton can provide a quick idea of whether the restoration criteria applied are adequate. In the case of La Pletera, the zooplankton indicator suggests that the restoration criteria used, based on the recovery of ecological functioning and the conservation of its characteristic hydrological dynamics (Quintana *et al.*, 2018), are appropriate.

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Reproductive strategies of *Moina* (Cladocera) in relation to their habitat

S. Nandini* and S.S.S. Sarma

Laboratory of Aquatic Zoology, Division of Investigation and Postgraduate Studies, UMF Building, National Autonomous University of Mexico, Campus Iztacala, AP 314, CP 54090, Tlalnepantla, State of Mexico, Mexico FAX: +52 55 5623 1256.

* Corresponding author: nandini@unam.mx

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ABSTRACT

Reproductive strategies of *Moina* (Cladocera) in relation to their habitat

Some *Moina* species are predominantly found in large ponds and lakes while others are restricted to temporary pools or estuaries. The life history strategies and resistance to starvation of different species of *Moina* depend on their habitat. We compared the demography and starvation resistance in three species of the genus: *Moina macrocopa* (isolated from a reservoir), *M.* cf. *micrura* (from a small lake) and *M.* cf. *wierzejskii* (from a temporary pool). Population growth of the three *Moina* species was followed for 15 days using *Chlorella vulgaris* at a density of 1 x 10^6 cells/ml. Daily the females, males and ephippia produced by each cladoceran species were enumerated and transferred to a fresh test medium. We also tested the differences in resistance to starvation of the adults and the neonates by comparing the days until all the unfed individuals had died. Population growth curves of *Moina macrocopa*, *M.* cf. *micrura* and *M.* cf. *wierzejskii* showed significantly different trends. *Moina macrocopa* had higher peak densities (5-6 ind/ml) and higher population growth rates (0.33 per day) than the other two species. However, *M.* cf. *wierzejskii* had higher production of both, males and ephippia (0.3-0.4 ind/ ml and 0.7-0.8 ind/ml, respectively) than the rest. Regardless of the species, neonates were less resistant to starvation than adults. We discuss here adaptations of the life history strategies of these taxa in relation to their habitat.

Key words: Crustacea, males, ephippia, reproduction, starvation

RESUMEN

Estrategias reproductivas de Moina *(Cladócera) en relación con su hábitat*

Algunas especies de Moina *se encuentran predominantemente en grandes estanques y lagos, mientras que otras están restringidas a estanques temporales o estuarios. Las estrategias de vida y la resistencia al hambre de diferentes especies de* Moina *dependen de su hábitat. Comparamos la demografía y la resistencia al hambre en tres especies del género:* Moina macrocopa *(de un embalse) y* M. *cf.* micrura *(aislado de un pequeño lago) y* M. *cf.* wierzejskii *(de un cuerpo de agua temporal). Se siguió el crecimiento poblacional de las tres especies de* Moina *durante 15 días utilizando* Chlorella vulgaris *a una densidad de 1 x* 10^6 *células/ml como alimento. Diariamente se enumeraron las hembras, machos y ephippia producidos por cada especie de cladócero y se transfirieron a un medio fresco. También probamos las diferencias en la resistencia a la inanición de los adultos y los recién nacidos al comparar los días hasta que todos los individuos sin alimento habían muerto. Las curvas de crecimiento poblacional de* Moina macrocopa, M. *cf.* micrura *y* M. *cf.* wierzejskii *mostraron tendencias claramente diferentes.* Moina macrocopa *tuvo mayores densidades máximas (5-6 ind./ml) y mayores tasas de crecimiento poblacional (0.33 por día) que las otras dos especies. Sin embargo,* M. *cf.* wierzejskii *tuvo una mayor producción de ambos, machos y ephippia, (0.3-0.4 ind/ml y 0.7-0.8 ind/ml, respectivamente) que al resto de las especies. Independientemente de la especie, los neonatos eran menos resistentes que los adultos. Discutimos aquí las adaptaciones de las estrategias de historia de vida de estos taxones con relación a su hábitat.*

Palabras clave: *Crustacea, machos, ephippia, reproducción, inanición*

INTRODUCTION

Cladocerans are found in diverse habitats ranging from permanent water bodies such as rivers, lakes and oceans to temporary water bodies such as rain pools and shallow ponds (Smirnov, 2017). Though predominantly parthenogenetic, when subject to stress, they produce males and ephippia. Switching from parthenogenesis to gametogenesis in cladocerans is induced by various factors including temperature, photoperiod, population density, and predator kairomones (Dodson & Frey, 2001). Ephippia, which are viable for several years, are extremely important in ensuring the survival of the population, especially in shallow pools subject to complete drying where the active egg bank is more than the inactive one (Cáceres & Hairston, 1998; Burge *et al.*, 2018).

In spite of the fact that there are more than 700 species of Cladocera (Smirnov, 2017), most of the ecological studies have focused on the family Daphniidae, particularly various species of the genus *Daphnia*. The family Moinidae has about 29 species worldwide of which seven are known from the Nearctic region (Forró *et al.*, 2008). Most species of *Moina* inhabit freshwater bodies around the world and are particularly abundant in the tropics and sub-tropics. While some are predominantly found in large ponds and lakes, others are restricted to temporary pools (Błędzki & Rybak, 2016). The life history strategies of species vary depending on the permanency of the habitat where they are found (Stearns, 1992).

In cyclical parthenogens including *Moina*, males are produced when the population reaches a critical density where there are sufficient females for random male-female encounters leading to the formation of ephippia (Winsor & Innes, 2002). Production of resting stages lowers the population growth rates of zooplankton (Brendonck *et al.*, 2017). However, species inhabiting temporary water bodies need to ensure the formation of resting stages and therefore should invest more in male and ephippial production than those that inhabit permanent waters (Dodson & Frey, 2010). Taxa living in temporary habitats with an uncertain supply of food resources should also be more resistant to starvation (Gross, 2012). Here, using three species of the same genus, *Moina*, we tested the hypothesis that species (*M.* cf. *wierzejskii*) isolated from temporary water bodies would invest earlier in male production than those (*Moina macrocopa* and *M.* cf. *micrura*) found in permanent ponds and lakes and would be more resistant to starvation.

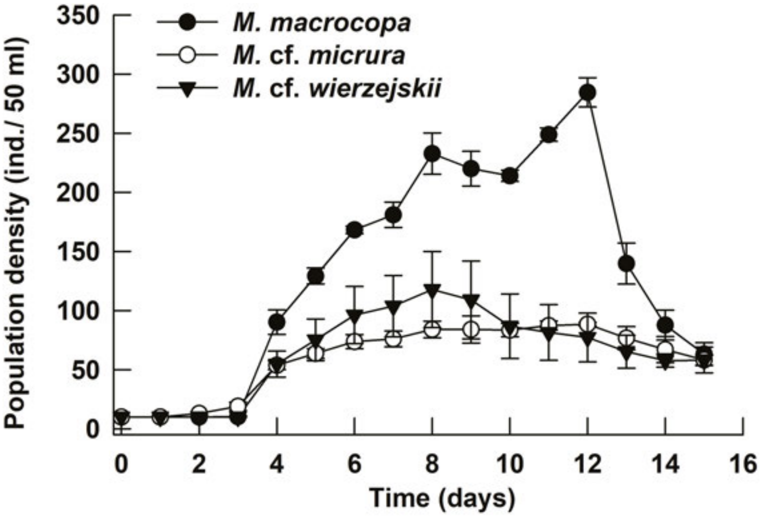

Figure 1. Population growth of *Moina* spp. in relation to culture time. Shown are the mean±standard error based on four replicates. *Crecimiento poblacional de* Moina *spp. en relación al tiempo. Se muestra la media ± error estándar basado en cuatro réplicas.*

MATERIAL AND METHODS

We isolated *Moina macrocopa americana* Goulden, 1968 from Lake Valsequillo (18° 55' 0" N and 98° 10' 0" W) in the State of Puebla, Mexico, *M.* cf. *micrura* Kurz, 1874 from Espejo de los Lirios Lake in the State of Mexico (19° 39' 2" N, Longitude: 99° 13' 19" W) and *M.* cf. *wierzejskii* Richard, 1895, from a shallow temporary water-body near Xochimilco Lake (19° 16' 30" N 99° 08' 20" W) in Mexico City. Clonal cultures for each cladoceran species were separately established using a single female on synthetic moderately hard water (EPA medium). We prepared the EPA medium by dissolving 96 mg $NaHCO_3$, 60 mg $CaSO_4$, 60 mg $MgSO_4$ and 4 mg KCl in one liter of distilled water (Weber, 1993). For food, we used *Chlorella vulgaris* at a density of 1 x 10^6 cells/ml. In terms of carbon, this was within the food level (4 mg/l C) recorded in eutrophic waters of Central Mexico (Enríquez García *et al.*, 2003). *Chlorella* was batch-cultured in 2L bottles using Bold's basal medium (Borowitzka & Borowitzka, 1988). Log phase alga was harvested by centrifuging at 2000 rpm for three minutes, then rinsed and re-suspended in a small quantity (10 ml) of distilled water. The concentrated alga was stored in a refrigerator (4 °C) until use. Algal density was estimated using a haemocytometer. The desired algal level (1 x 10^6 cells/ml) was obtained by diluting with EPA medium just before setting up the experiment.

Population growth experiments were conducted, separately for each *Moina* species, in 100 ml transparent jars containing 50 ml EPA medium and the chosen algal food density. Into each container, we introduced ten individuals of one of the three *Moina* species under a stereomicroscope using a Pasteur pipette. For each treatment, we set up four replicates. The test jars were maintained in a temperature controlled biological incubator set at 24±1 °C. The pH of the test medium was near neutral (7.2) and photoperiod in a 24 h cycle was set at 12h: 12h L: D. Following initiation of growth experiment, daily we counted the number of females, males and ephippia produced and returned them to fresh jars containing EPA medium with chosen algal food. The experiments were continued for 15 days by which time population densities of the three test species began to decline in each jar.

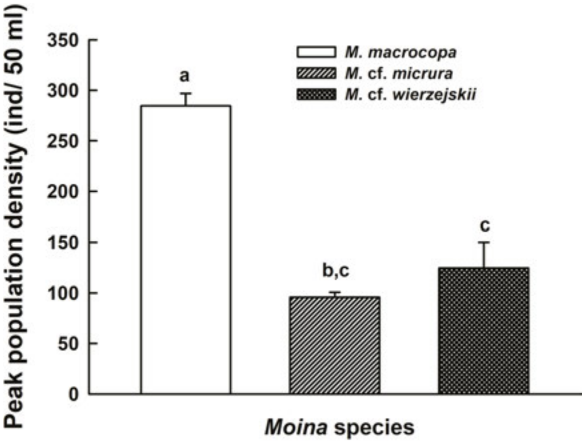

Figure 2. Peak population densities of *Moina* spp. cultured on *Chlorella vulgaris* at a density of 1 x 10^6 cells/ml). Shown are mean±standard error based on four replicates. Data bars carrying similar alphabets are not statistically significant ($p > 0.05$, Tukey test). *Las densidades máximas de población de* Moina *spp. cultivado en* Chlorella vulgaris *a una densidad de 1 x* 10^6 *células/ml. Se muestra la media ± error estándar en base a cuatro repeticiones. Las barras de datos con alfabetos similares no son estadísticamente significativas (*p *> 0.05, prueba de Tukey).*

To test the differences in resistance to starvation of the adults and the neonates we placed ten adults and ten neonates of each species in different jars containing EPA medium but no food. Four replicates per treatment and per species were used. We counted the number of individuals alive in each jar daily and returned them to new jars containing fresh medium. Dead individuals, when present, were counted and discarded. The experiment lasted three days by which time most individuals were dead in each jar.

From the population growth data, we derived rate of population increase per day using the following exponential growth equation (Krebs, 1985): $r = (\ln N_t - \ln N_o)/t$, where: r = rate of population growth, N_o = initial population density, N_t = final population density, and t = time in days.

The differences in the rate of population increase and maximal population abundances of the tested species were analyzed using a one-way ANOVA and Tukey's test (Statistica version 5).

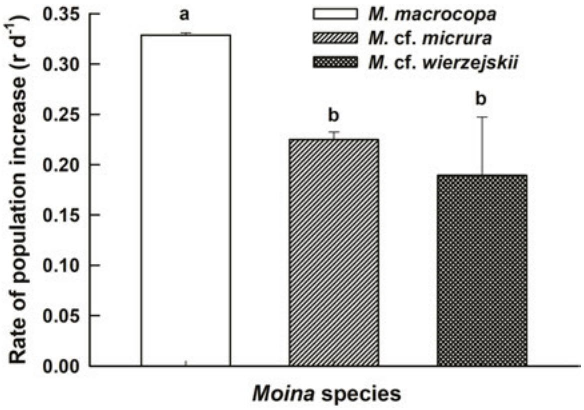

Figure 3. Rate of population per day (*r*) of *Moina* spp. cultured on *Chlorella vulgaris* at a density of 1 x 10^6 cells/ml. Shown are mean±standard error based on four replicates. Data bars carrying similar alphabets are not statistically significant ($p > 0.05$, Tukey test). *Tasa de crecimiento poblacional por día (r) de* Moina *spp. cultivado con* Chlorella vulgaris *a una densidad de 1 x 10^6 células/ml. Se muestra la media ± error estándar en base a cuatro repeticiones. Las barras de datos con alfabetos similares no son estadísticamente significativas (*p *> 0.05, prueba de Tukey).*

RESULTS

Population growth curves of *Moina macrocopa americana* (here after as *M. macrocopa*), *M.* cf. *micrura* and *M.* cf. *wierzejskii* (Fig. 1) showed that all the three species entered the exponential growth phase after day 3. However, *M. macrocopa* continued to grow until day 12, and after that, there was a steep fall in its abundances. On the other hand, both *M.* cf. *micrura* and *M.* cf. *wierzejskii* showed similar growth trends where after one week the populations began to decline. *Moina macrocopa* reached higher densities than the rest; *M.* cf. *micrura* had the least peak population abundance. The peak population abundances of the three *Moina* species ranged from 100 to 27 ind. 50 ml^{-1} and were significantly different ($p < 0.05$, One way ANOVA, Table 1). However, pairwise comparisons using post hoc tests showed that the peak population densities of *M.* cf. *micrura* and *M.* cf. *wierzejskii* were not significantly different ($p > 0.05$) (Fig. 2). The rate of

Table 1. Results of one-way ANOVA performed for peak population density, rate of population increase, total male density and total ephippial density among *Moina macrocopa*, *M.* cf. *micrura* and *M.* cf. *wierzejskii*. DF: Degrees of Freedom; SS: sum of square; MS: mean- square, F: Fisher´s ratio. *Resultados del ANOVA realizado para la densidad poblacional máxima, la tasa de crecimiento poblacional, la densidad total de los machos y la densidad de las ephippias total entre* Moina macrocopa*,* M. *cf.* micrura *y* M. *cf.* wierzejskii. *DF: Grados de libertad; SS: suma del cuadrado; MS: mean-square, F: Fisher's ratio.*

Source of Variation	DF	SS	MS	F	*p*
Peak population density					
Between groups	2	82 650	41 325	37.84	<0.001
Error	9	9828	1092		
Rate of pop. increase					
Between groups	2	0.042	0.021	4.63	<0.05
Error	9	0.041	0.004		
Male density					
Between groups	2	42 643	21 321	11.81	<0.01
Residual	9	16 247	1805		
Ephippial density					
Between groups	2	93 020	46 510	57.06	<0.001
Error	9	7336	815		

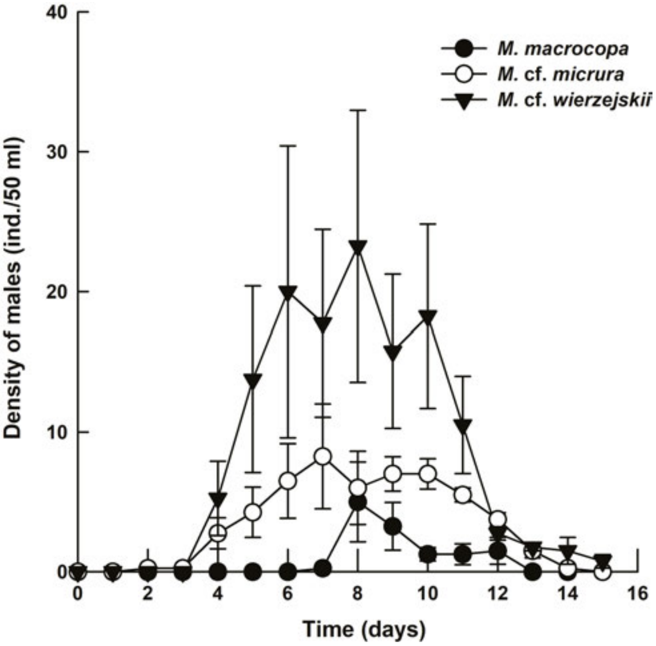

Figure 4. Dynamics of male production by *Moina* spp. in relation to culture period. Shown are the mean±standard error based on four replicates. *Dinámica de la producción de machos por* Moina *spp. en relación con el período de cultura. Se muestra la media ± error estándar en base a cuatro réplicas.*

population increase per day of the three *Moina* species varied from 0.19 to 0.33, with the highest for *M. macrocopa*. Statistically, the *r* values were significantly different among the three cladoceran species ($p < 0.001$, F test). However, pairwise comparisons showed that the *r* of *M.* cf. *micrura* was not significantly different from *M.* cf. *wierzejskii* ($p > 0.05$, Tukey test, Fig. 3).

Dynamics of male production by the three species of *Moina* is shown in Fig. 4. In general, we found a higher number of males in *M.* cf. *wierzejskii* as compared to the other two *Moina* species. The differences in the total male production as well as the peak abundances of males were statistically significant ($p < 0.01$, one way ANOVA, Table 1) (Fig. 5). Few ephippia were produced by *M. macrocopa*, while for *M.* cf. *micrura* and *M.* cf. *wierzejskii* the mean total of ephippial density was 100 and 215 numbers per jar, respectively (Fig. 6).

Regardless of the species, neonates of *Moina* are less resistant than adults (Fig. 7). Both neonates and adults of the three tested species did not survive beyond four days of starvation. How-

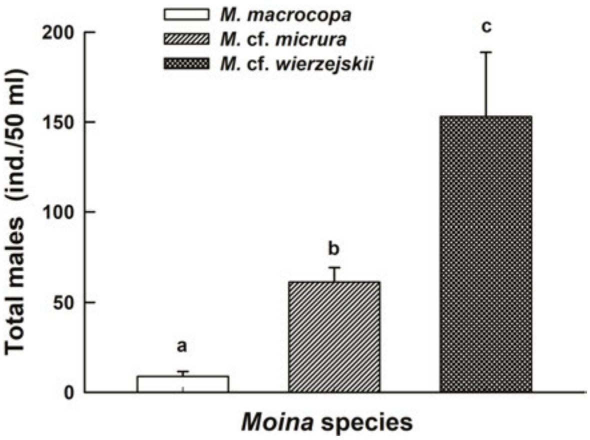

Figure 5. Total males produced by different *Moina* spp. cultured on *Chlorella vulgaris* at a density of 1 x 10^6 cells/ml. Shown are the mean±standard error based on four replicates. Data bars carrying dissimilar alphabets are statistically significant ($p < 0.05$, Tukey test). *Machos totales producidos por diferentes* Moina *spp. cultivado en* Chlorella vulgaris *a una densidad de 1 x* 10^6 *células/ml. Se muestra la media ± error estándar en base a cuatro réplicas. Las barras de datos que llevan alfabetos diferentes son estadísticamente significativas (*p *< 0.05, prueba de Tukey).*

ever, adults of *M.* cf. *wierzejskii* showed better survival until the third day than *M. macrocopa* and *M.* cf. *micrura*.

DISCUSSION

Reproductive characteristics of cladocerans, including *Moina*, are considered from population growth rates, age, size at first reproduction and clutch size (Stearns, 1992). Although essential, these cannot adequately explain adaptation to harsh environmental conditions. For example, when conditions are not favourable, most cladocerans switch to male, followed by, ephippia production (Alekseev & Lampert, 2001). When ponds and other temporary waterbodies dry up, the only surviving forms of cladocerans are the ephippia (Gerhard *et al.*, 2017). *Moina* spp. are adapted to living in both temporary and permanent waters. In seasonally drying ponds or, ponds, which experience a high degree of environmental changes such as temperature and food levels, *Moina* cf. *wierzejskii* is common (Goulden, 1968). On the other hand, species like *M. macrocopa* and *M.* cf. *micrura* occur in ponds and lakes which do not dry completely (Bledzki & Rybak, 2016). Therefore, their reproductive and survival characteristics reflect their adaptations to the conditions in which they naturally occur.

It is believed that cladoceran genera such as *Daphnia* and *Moina* reproduce parthenogenetically when conditions are favourable, and males and ephippia appear only when conditions are not favourable (Dodson & Frey, 2010). In the present study, this was also the case for *M. macrocopa* and *M.* cf. *micrura*. However, for *Moina* cf. *wierzejskii*, both male and ephippia production started within a week even when the conditions were favourable which has an adaptive advantage for this species (Zadereev, 2003). When the conditions are unpredictable, a greater investment in parthenogenetic reproduction alone does not ensure the survival of the species (Jiménez & Zoppi de Roa, 1987). If a species invests a portion of its assimilated energy into the production of males when the resources are

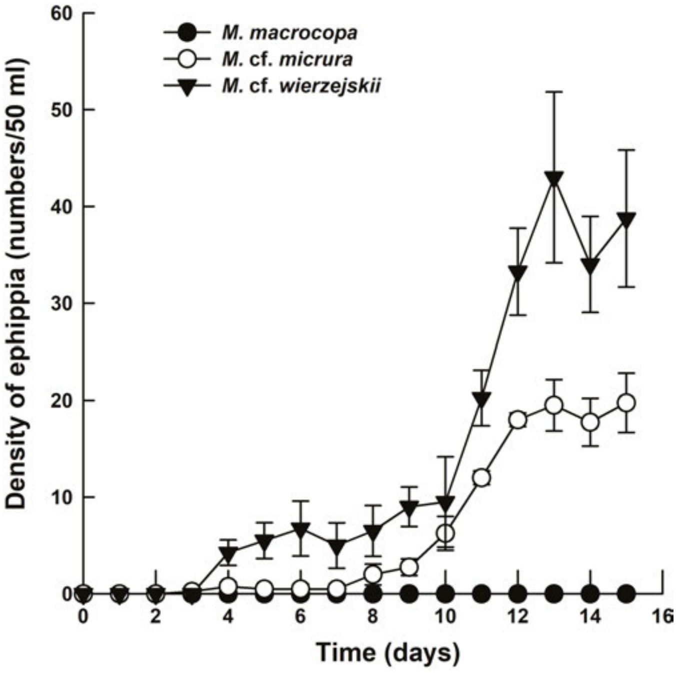

Figure 6. Dynamics of ephippial production by *Moina* spp. in relation to culture period. Shown are the mean±standard error based on four replicates. *Dinámica de la producción de ephippial por* Moina *spp. en relación con el período de cultura. Se muestra la media ± error estándar en base a cuatro réplicas.*

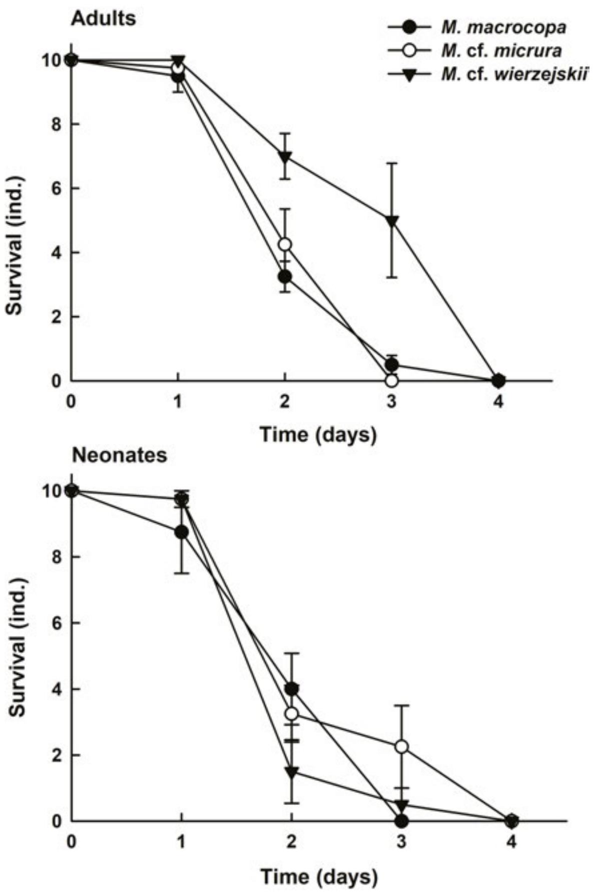

Figure 7. Resistance to starvation by *Moina* spp. adults and neonates. Shown are the mean±standard error based on four replicates. *Resistencia al hambre por* Moina *spp. adultos y recién nacidos. Se muestra la media ± error estándar en base a cuatro réplicas.*

not limiting or when the conditions are not yet harsh enough, then viable ephippial numbers are guaranteed which would ensure population buildup when favourable conditions return. On the other hand, since both *M. macrocopa* and *M.* cf. *micrura* are adapted to nearly predictable environmental conditions, production of males is not desirable since the production of males and ephippia results in lower population growth rates (Sommer *et al.*, 2016). It thus seems that *M.* cf. *wierzejskii* invests its energy in early male and ephippial production to ensure a seed bank even when the conditions are appropriate for parthenogenetic reproduction. This species, often found in small, shallow, rain-fed pools, is also capable of reaching critical densities earlier which could result in successful male-female encounters and ephippia production (Carvalho & Hughes, 1983). High male-producing clones become extinct faster in daphniids (Innes & Singleton, 2000); we also observed this here when *M.* cf. *wierzejskii* began to decline earlier other *Moina* species tested. However, by producing the ephippia earlier, this species ensures its survival in unpredictable or temporary environments (Hairston & Cáceres, 1996). Delaying sexual reproduction influences fitness differentially, depending on the habitat. In permanent waters, investment in parthenogenesis will ensure more individuals and a higher success at sexual reproduction due to more male-female encounters. In temporary habitats, however, delaying sexual reproduction will lower the fitness since the habitat may disappear (Burke & Bonduriansky, 2018).

Resistance to starvation is yet another essential characteristic of populations living under unpredictable or highly fluctuating environmental conditions. In general, longer the period of resistance to starvation, higher is the possibility of utilizing a future food source (Kirk, 2012). The fact that *M.* cf. *wierzejskii* showed higher survival of the population than the other two species implies its adaptation to unpredictable environmental conditions where daily food availability is not guaranteed.

Moina macrocopa has higher lifespan and population growth rates as compared to *M.* cf. *wierzejskii* (Nandini & Sarma, 2006). Here too we show that *M. macrocopa* had higher growth rates than *M.* cf. *wierzejskii*. Previous studies indicate that the threshold food concentration of cladocerans is inversely or curvilinearly related to their body size (Gliwicz 1990; Nandini & Sarma, 2006). *Moina macrocopa* has a higher threshold food concentration than *Daphnia laevis* (Nandini & Sarma, 2003), probably because it is not as efficient as *Daphnia* in filtering food as a result of the density of setules on the thoracopods (Monakov, 2003; Smirnov, 2017). It is possible that competition between the three species would result in *Moina macrocopa* displacing the other two taxa. However, we rarely found all three species coexisting in the same habitat.

CONCLUSIONS

Our study thus showed that though the three *Moina* species were cultured under similar conditions, they differed in their population growth characteristics, male and ephippial production suiting the habitats to which they are adapted. These differences in life history strategies explain their physical separation from each other too.

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Evidence does not support the conclusion that *Hexarthra intermedia* (Rotifera, Monogononta, Flosculariaceae) causes mortality in *Bosmina longirostris* (Cladocera, Diplostraca, Branchiopoda)

Robert L. Wallace[1,*] and Elizabeth J. Walsh[2]

[1] Department of Biology, Ripon College, Ripon, WI, 54971, USA.
[2] Department of Biological Sciences, 500 W. University Ave., University of Texas at El Paso, El Paso, TX, 79968, USA.

[*] Corresponding author: wallacer@ripon.edu

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ABSTRACT

Evidence does not support the conclusion that *Hexarthra intermedia* (Rotifera, Monogononta, Flosculariaceae) causes mortality in *Bosmina longirostris* (Cladocera, Diplostraca, Branchiopoda)

Based on observations of preserved samples, Jaramillo-Londoño & Pinto-Coelho (2010) describe a putative ecological relationship between the rotifer *Hexarthra intermedia* and the cladoceran *Bosmina longirostris* as a fusion of parasitism and interference competition. They argue that *H. intermedia* enter the filtering chamber of *B. longirostris* allowing the rotifer to exploit food collected by *Bosmina*, while simultaneously receiving protection from predators. In addition, they surmise that the result of this interaction can lead to death of *Bosmina*. We posit two simpler explanations for their observations. (1) Presence of *H. intermedia* in the cladoceran's filtering chamber is an artifact of the collection methods. (2) Statistical correlations between the population levels of these two species simply means that the environment was suitable for their growth. Thus until additional evidence corroborates their findings, the simpler explanations should prevail.

Key words: freshwater, inference competition, María Rosa Miracle, parasitism, zooplankton

RESUMEN

La evidencia no soporta la conclusión de que* Hexarthra intermedia *(Rotifera, Monogononta, Flosculariaceae) es la causa de la mortalidad de* Bosmina longirostris *(Cladocera, Diplostraca, Branchiopoda)

Jaramillo-Londoño y Pinto-Coelho (2010) describen una relación ecológica putativa entre el rotífero Hexarthra intermedia *y el cladócero* Bosmina longirostris *como una fusión de parasitismo y competencia por interferencia con base en observaciones hechas en muestras preservadas. Se argumenta que* H. intermedia *ingresa a la cámara de filtración de* B. longirostris *permitiendo al rotífero aprovechar los alimentos recolectados por* Bosmina*, y al mismo tiempo recibir protección de los depredadores. El resultado de esta interacción puede llevar a la muerte de* Bosmina*. Postulamos explicaciones más simples para sus observaciones. (1) La presencia de* H. intermedia *en la cámara de filtrado del cladócero es un resultado del método de recolección del mismo. (2) Las correlaciones estadísticas entre los niveles de población de estas dos especies simplemente significan que el ambiente era adecuado para su crecimiento. Por lo tanto, hasta que la evidencia adicional corrobore sus hallazgos, las explicaciones más simples deben prevalecer.*

Palabras clave: *agua dulce, competencia por interferencia, María Rosa Miracle, parasitismo, zooplancton*

Preface

This paper, along with the others appearing in this volume, lauds the meticulous research of Prof. Dr. María Rosa Miracle. During her distinguished career spanning five decades she greatly expanded our understanding of the ecological niche of rotifers (Miracle, 1974; Carmona *et al.*, 1989; Esparcia *et al.*, 1989; Vicente & Miracle, 1992; Miracle *et al.*, 1995; Armengol & Miracle, 1999; Miracle *et al.*, 2007; Miracle *et al.*, 2014; Onandia *et al.*, 2015). Her thorough work should serve as a guide to other investigations. Here we emphasize the care that must be taken in studies that explore interactions of rotifers with other zooplankton species and in drawing inferences that expand the rotiferan niche.

INTRODUCTION

Understanding community structure and population dynamics of zooplankton, as well as the details of their biotic interactions, requires studies across several scales, both spatial and temporal. At large spatial scales studies have examined zooplankton in many freshwaters systems. These kinds of studies inform us about how edaphic conditions influence zooplankton community composition and development (Balayla *et al.*, 2010; Obertegger *et al.*, 2010; Pinel-Alloul & Mimouni, 2013; Hiltunen *et al.*, 2015; Gozdziejewska *et al.*, 2016). Study at small spatial scales revealed the patchiness of the plankton (Lehman & Scavia 1982; Schuler *et al.*, 2017), as well microhabitat preferences among species (Walsh, 1995; Van de Meutter *et al.*, 2004; Kuczynska-Kippen & Nagengast, 2006). Research across long-term temporal scales (years to decades) has uncovered information about the dynamics of populations yielding information about when species appear and disappear seasonally, yearly, or longer (Herzig, 1987; Hampton *et al.*, 2006; Hampton & Schindler, 2006; Muirhead *et al.*, 2006; Smith *et al.*, 2009; Obertegger *et al.*, 2011; Francis *et al.*, 2014). At intermediate timescales (weeks to months) investigations have provided details about individual species or a suite of similar species (Ruttner-Kolisko, 1977; Fey *et al.*, 2010; Sastri *et al.*, 2014; McMeans *et al.*, 2015). At the smallest timescales (minutes to days) we have learned some of the details of how individual species interact with each other and their abiotic environment (Burns & Gilbert, 1986; Kirk & Gilbert, 1988; Kirk & Gilbert 1990; Hampton & Gilbert, 2001; Diéguez & Gilbert 2011; Sarma *et al.*, 2011). Thus, from the collective research across these scales several influential techniques and concepts have been established including, for example, the egg ratio technique, the microbial loop, community hysteresis and compensatory dynamics in zooplankton communities, feeding specializations, and other unique behaviors. Of course, the weight of these concepts has been amplified by careful observation and critical analysis. More importantly, these concepts have been confirmed by repeated observations and experimentation.

Edmondson's egg ratio technique has been used to analyze the population dynamics of a variety of zooplankton populations (Edmondson, 1960; Edmondson *et al.*, 1962; Edmondson, 1968; Balayla & Moss, 2003). Other researchers subsequently modified the technique; the collective outcome was an important tool for study of *in situ* population dynamics (Caswell, 1972; Paloheimo, 1974). Elucidation of the microbial loop gave new insight into energy flow and nutrients cycling in aquatic systems (Azam *et al.*, 1983; Stockner & Porter, 1988; Arndt, 1993; Jürgens & Jeppesen, 2000; Azémar *et al.*, 2006; Kissman *et al.*, 2017). Careful examination of zooplankton community structure during perturbations has led to a better understanding of both hysteresis and compensatory dynamics (e.g., Fischer *et al.*, 2001; Frost *et al.*, 2006; Pace *et al.*, 2013). Several accounts have documented the concept of feeding specialization in zooplankton. Actually understanding feeding specialization in these taxa has been an important area of research for many years (Wallace *et al.*, 2006 and references therein). Undeniably, the field is replete with studies that have explored this subject, either in field or laboratory settings (e.g., Edmondson, 1965; Bodgan & Gilbert, 1984; Stemberger & Gilbert, 1987; Williamson & Vanderploeg, 1988; Brett & Müller-Navarra, 1997; Jackson & Thomas Kiørboe, 2004; Noyon & Froneman, 2014; Onandia *et al.*, 2015; Benedetti *et al.*, 2016).

Research also has documented the occurrence of interesting trophic interactions among protists and micrometazoans (i.e., cladocerans, fungi, and rotifers). Here we note four examples. (1) Ciliates and heliozoans engulf whole rotifers (Wallace *et al.*, 2015). (2) Soil and aquatic fungi feed as saprophytic parasites on rotifers and nematodes (Barron, 1980; Robb & Barron, 1982; Barron & Szijarto, 1984; Fialkowska & Pajdak-Stós, 2018). (3) Rotifer species of *Dicranophorus* and *Proales* are carrion feeders, cleaning out the carapaces of dead cladocerans and copepods, as well as the bodies of freshwater oligochaetes (Nogrady *et al.*, 1993; Wallace *et al.*, 2006). (4) Rotifers also are predatory on cladocerans. The rotifer *Dicranophorus isothes* Harring & Myers, 1928 is a predator, feeding strictly on cladocerans such as *Alona*, *Chydorus*, and small *Moina*. Harring & Myers (1928) provide a brief description of this rotifer's

Table 1. An analysis of the argument presented by Jaramillo-Londoño and Pinto-Coelho (2010) regarding interactions between *Hexarthra intermedia* and *Bosmina longirostris*. *Un análisis del argumento presentado por Jaramillo-Londoño y Pinto-Coelho (2010) sobre las interacciones entre* Hexarthra intermedia *y* Bosmina longirostris*.*

Our comment number (Original Pagination)	Synopsis of the authors' points	Our remarks
1 (962)	Samples were preserved in 4% formalin and transported back to the laboratory for analysis.	No chemical agents were added to reduce potential for osmotic shock. No anesthetization was attempted. Preserved animals can become agitated during transportation.
2 (963)	≤4 well-preserved *Hexarthra* were present inside empty carapaces of *Bosmina*; see Fig. 3B.	Distortion is common during preservation of cladocerans (e.g., the body pulls away from the carapace). *Hexarthra* may then enter an empty carapace.
3 (963)	*Hexarthra* density was positively correlated with both density of *Bosmina* and with their exuviae, suggesting existence of a recurrent association and also the possible impact of *Hexarthra* on *Bosmina* death rates.	Statistical correlation does not necessarily mean causation. An alternative explanation that could be proposed is that both species are responding to favorable conditions (e.g., availability of food, temperature, and few predators).
4 (964)	*Hexarthra* has malleoramate trophi, prefers food sizes of <6 µm. It moves with darting a motion that results in *Bosmina* becoming prey.	These two sentences comprise a 3-part syllogism. The logical fallacy that this argument presents is discussed in the body of our text.
5 (964)	*Hexarthra* in the filtering chamber of *Bosmina* suggests a fusion of parasitism with interference competition.	No evidence has been provided to document live *Hexarthra* entering the thoracic region of *Bosmina* where suspension feeding takes place.
6 (964)	*Hexarthra* causes noticeable damage to the "hosts", this relationship also approaches some form of parasitism (Fig. 5).	No evidence has been provided that qualifies as noticeable damage having been inflicted on the *Bosmina*.

predatory activities. NB: A permanent slide deposited in The Academy of Natural Sciences of Philadelphia [ANSP] (now The Academy of Natural Sciences of Drexel University) and pictured in Jersabek *et al.* (2003) depicts a putative predatory event between *D. isothes* and *Alona* sp. (F.J. Myers is credited with the preparation of this slide [Specimen Preparation ANSP 912]). Also the rotifer *Asplanchnopus multiceps* (Schrank, 1793) is known to feed on cladocerans and rotifers (Nandini & Sarma, 2005). Other unusual feeding habits exhibited by rotifers are reviewed in Wallace *et al.* (2006).

However, another novel trophic pathway has been reported that requires additional analysis. Jaramillo-Londoño & Pinto-Coelho (2010) report that the rotifer *Hexarthra intermedia* (Wiszniewski, 1929) [hereafter *Hexarthra*] interact with the limnetic cladoceran *Bosmina longirostris* (O.F. Muller, 1785) [hereafter *Bosmina*], ultimately resulting in mortal damage being inflicted on the cladoceran. As of this writing we are aware of > 12 papers that have cited this paper, but most only reference it without commenting on its findings (e.g., Santos-Wisniewski *et al.*, 2011; Jaramillo-Londoño & Aguirre-Ramírez, 2012; Pauwels *et al.*, 2014; Kotov & Fuentes-Reines, 2015; Ergönül *et al.*, 2016; Gürbüzer *et al.*, 2017). Because observation regarding *Hexarthra* impacting *Bosmina* influences our understanding of the freshwater food web, in general, and specifically the niche and trophic dynamics of rotifers, we believe that the methods and conclusions of this paper need to be re-examined carefully. In the spirit of Miracle's careful attention to detail, we review the methodology, results, and conclusions of Jaramillo-Londoño & Pinto-Coelho (2010) and offer alternative explanations to their observations.

EVIDENCE AND A REINTERPRETATION

The research of Jaramillo-Londoño & Pinto-Coelho (2010) was based on preserved zooplankton samples taken from the Vargem das Flores Reservoir, located ca. 20 km southwest of Belo Horizonte, Minas Gerais, Brazil. The unusual conclusion proposed in this paper is that *Hexarthra* exploit a new trophic-ecological niche axis (page 964) representing "… a fusion of parasitism with interference competition." The implied logic is that the food for *Hexarthra* in this reservoir is composed mainly of small coccoid shaped cells, which provide only some of the dietary needs for this rotifer. These authors hypothesized that *Hexarthra* is able to find additional nutritional resources by intruding into the filtering chamber of *Bosmina*, thereby exploiting resources (algal particles filtered by *Bosmina* and residing in its filtering chamber). In addition, while in the filtering chamber *Hexarthra* also receives shelter from predators. We highlight six instances where the evidence provided is insufficient to support the authors' conclusions (Table 1). In presenting our analysis (below) we quote the authors.

Point 1 (Page 962). "All samples were preserved in 4 % formalin for further laboratory processing." The statement gives no indication whether their preservation methods followed the standard protocol for preserving plankton samples as described by Downing & Rigler (1984). As these researchers point out, unless care is taken cladocerans can become distorted (ballooning). Ballooning often results in a forward flexure of the abdomen, which exposes the brood chamber and permits loss of developing young (see Haney & Hall, 1973). Thus poor preservation may be the cause of the animal's flexing seen in Fig. 1B. The result of distortion of the *Bosmina* specimens during fixation may be, in part, responsible for *Hexarthra* ending up in the filtering chamber of *Bosmina* in the preserved samples.

Point 2 (Page 963). "It was common to detect up to four well-preserved individuals of *H. intermedia* inside empty bodies of *B. longirostris*." It is well known by researchers who study *Hexarthra* that they frequently adhere to one another and to other materials in preserved samples – sometimes even the arms adhere to the bodies of other rotifers (S. Nandini, S.S.S. Sarma, & R. Shiel, pers. comm.; EJW, pers. obs.). This fact can account for the connection of *Hexarthra* to live *Bosmina* that were killed during preservation, as well as to their exuviae. With sufficient agitation during transport to the laboratory the spine-bearing arms of *Hexarthra* could easily become entangled with *Bosmina*.

Point 3 (Page 963). "The density of *H. intermedia* was positively correlated with the density of *B. longirostris* containing *H. intermedia* … , and with the occurrence of carapaces of *B. longirostris* containing *H. intermedia* … (Fig. 4)." If the carapaces are exuviae, then the occurrence of *Hexarthra* within them may well be simply circumstantial. "These results and correlations suggest not only the existence of a recurrent association between these two species, but also the possible impact of *H. intermedia* intrusions on death rates of *B. longirostris*." A positive correlation between two factors does not mean cause and effect. Given that both species are suspension feeders, a correlation of population sizes probably indicates that they are responding to favorable conditions in the habitat. That is, there is adequate food and probably also favorable temperature and few predators. This association does not confirm cause and effect of an "… impact of *H. intermedia* intrusions on death rates of *B. longirostris*."

Point 4 (Page 964). One paragraph, comprising two sentences, essentially constitutes a three-part categorical syllogism, which may be presented as follows.

1. "*Hexarthra intermedia* has a mastax with malleoramate trophi … , and prefers [to consume] particles smaller than 6 μm … ."
2. "Its darting movements [of *Hexarthra*] offer some protection against predation … , and"
3. "so [due to the darting movements of *Hexarthra*] *B. longirostris* becomes an easy prey to capture … ."

Unfortunately, the elements in this syllogism are not logically connected. The fact that *Hexarthra* has malleoramate trophi has nothing to do with the fact that this rotifer can move by darting movements (jumps). While frequent jumps by *Hexarthra* may increase encounters with other large planktonic organisms, they do not make *Bosmina* an easy prey. Indeed the darting movements or jumps of *Hexarthra* (and other rotifers such as *Polyarthra*) are escape movements that move the animals away from disturbances in the water, not toward them (Kirk & Gilbert, 1988; Kak & Rao, 1998; Hochberg & Ablak Gurbuz, 2008; Hochberg *et al.*, 2017). Thus, the rapid evasive movements of *Hexarthra* appear to protect it from being swept into the branchial chambers of cladocerans.

The statement that "… *B. longirostris* becomes an easy prey to capture … ." [by *Hexarthra*] is puzzling. The typical diet of *Hexarthra* is known to comprise particles in the size range of bacteria and algae (≤ 6 μm) (Bouvy *et al.*, 1994; Pagano *et al.*, 1998). These limits in food size are a function of morphology of its malleoramate trophi, which are used to process food by a grinding action (Fontaneto & De Smet, 2015). Additionally, the mastax (with trophi) lies deep enough inside *Hexarthra*'s body to prevent the animal from protruding its trophi through the mouth to grasp individual prey items. That sort of predatory behavior is seen in raptorial, predatory rotifers such as *Asplanchna* and *Asplanchnopus* (Wallace *et al.*, 2015; H. Segers, pers. comm.). Thus, *Hexarthra* is simply not mechanically suited to feed on large cladoceran prey. To do so would require extruding its trophi from its mouth, cutting thorough the exoskeleton, and then rending tissues for ingestion.

Point 5 (Page 964). The authors note that "… intrusion of individuals of *H. intermedia* in the filtering chamber of *Bosmina* is not a trivial feature, … ." The photomicrographs of *Hexarthra* and *Bosmina* (Figs. 1B-D) do not support this assertion. They show the following: Fig. 1B – one *Hexarthra* near the exit of the brood chamber and another attached to the legs; Fig. 1C – one *Hexarthra* in an empty carapace of *Bosmina*; and Fig. 1D – a small *Bosmina* in juxtaposition with a carapace from a larger animal, with one, perhaps two, *Hexarthra* also adhering to the carapace. The positioning could be a result of preservation artifacts as discussed above.

Heeg & Rayner (1988) also have described close associations among zooplankton in preserved samples. They reported *Hexarthra mira* (Hudson, 1871) forming short, conspecific chains of between two and seven individuals. In some chains *Trichocerca chattoni* (Beauchamp, 1907) were also present. They also reported that both *H. mira* and *T. chattoni* attached to *B. longirostris*. These observations are similar to those made by Jaramillo-Londoño and Pinto-Chelho (2010), and thus we conclude that their observations also are misinterpretations of preservation artifacts.

Point 6 (Page 964). The authors state "… intrusion of individuals of *H. intermedia* in the filtering chamber of *Bosmina* is not a trivial feature, since it reveals signs of a new ecological relationship that could be seen as a fusion of parasitism with interference competition. … … Nevertheless, since it causes noticeable damage to the "hosts", this relationship also approaches some form of parasitism (Fig. 5)." The authors offer no documentation of *Hexarthra* inflicting damage to *Bosmina*. Actually, studies of rotifer-cladoceran interactions have documented the reverse; rotifers that have been swept into the branchial chamber of large cladocerans are often damaged (sometimes lethally) (Fradkin, 1995). We conclude that the relationship conceptualized in Fig. 5 is not warranted.

DISCUSSION

The possibility for the novel feeding dynamic of *Hexarthra* outlined by Jaramillo-Londoño & Pinto-Coelho (2010) is intriguing, but premature. Indeed simpler interpretations of their observations are warranted. The observation that the population size of *Hexarthra* was correlated to that of *Bosmina* is a misinterpretation of statistical correlation; it does not support an argument for cause and effect. Photomicrographs of *Hexarthra* in contact with *Bosmina* and even inside empty carapaces could be artifacts of the collection methods and the fact that *Hexarthra* adheres to materials in preserving fluids. Of course, we know that cladocerans and rotifers do interact by exploitative and/or interference competition with cladocerans possessing the competitive advantage (Gilbert, 1985; 1989), and that anostracans can consume *Hexarthra* sp. (Starkweather, 2005). However, the evidence provided by Jaramillo-Londoño & Pinto-Coelho (2010) reports no competitive advantage of *Bosmina* over *Hexarthra*. We suggest that additional research should be undertaken to explore the potential for competition between these taxa.

We agree with the author's final conclusion: "The final question regarding the effects of *H. intermedia* on *B. longirostris* is the potential impact of this interaction on *Bosmina* populations. Resolving this question will require a more exhaustive study." We suggest that such an exhaustive study requires the systematic application of the criteria outlined by Platt (1964) for scientific problems. This means formulating multiple working hypotheses that are testable, refutable, and repeatable. In this case, the study must include meticulous observations of live *Hexarthra* in close association with live *Bosmina*. Thus, until further evidence is obtained the answer to the question as to whether a new trophic interaction occurs between these two species is no.

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Hypoxia extends lifespan of *Brachionus manjavacas* (Rotifera)

Terry W. Snell*, Rachel K. Johnston and Brande L. Jones

School of Biological Sciences, Georgia Institute of Technology, Atlanta, Georgia, 30332-2230, USA.

* Corresponding author: terry.snell@biosci.gatech.edu

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ABSTRACT

Hypoxia extends lifespan of *Brachionus manjavacas* (Rotifera)

Work by Maria Rosa Miracle's group in 1989 described the adaptation of natural *Brachionus plicatilis* populations to low oxygen in oxyclines of meromictic lakes. This adaptation was accomplished by slowing metabolism in hypoxia, altering reproductive schedules and extending generation times. Many years later, Miracle's work inspired us to examine whether hypoxia could extend rotifer longevity and reproduction in our study of the biology of aging. In this paper we show that exposure of rotifers to four days of an atmosphere of 1.6 % O_2 extended mean lifespan 107 % over controls in normoxia (20.9 % O_2), whose mean lifespan was 7.9 days. Alternating days of hypoxia and normoxia also markedly extended rotifer lifespan by 53 %. Exposure to 1.6 % O_2 hypoxia nearly doubled lifetime reproduction of females (24.3 vs 12.4 offspring in control). Hypoxia exposure protected rotifers from subsequent oxidative and UV stress, but not starvation, osmotic or heat stress. The 0-4 day age-classes responded best to hypoxia exposure as compared to hypoxia exposure days 4-8 or 8-12. The protective effects of two days of hypoxia exposure persisted through day 6, then vanished by day 8. Rotifer diapausing eggs are especially resistant to hypoxia and hatch in atmospheres containing as little as 1.6 % O_2 after a one day delay. Our conclusion is that exposure to hypoxia for four days in the youngest age classes extends rotifer longevity and enhances lifetime reproduction. This response has adaptive value in anoxic sediments where most rotifer diapausing eggs are deposited.

Key words: hypoxia, rotifers, Brachionus, lifespan

RESUMEN

La hipoxia alarga la vida media de Brachionus manjavacas *(Rotifera)*

En el trabajo realizado por Maria Rosa Miracle y colaboradores en 1989 se describió la adaptación de las poblaciones naturales de Brachionus plicatilis *a los bajos niveles de oxígeno propios de las oxiclinas de los lagos meromícticos. Esta adaptación se conseguía al desacelerar el metabolismo en la hipoxia, alterando su programa reproductivo y a los tiempos de generación. Muchos años después, el trabajo de Miracle nos inspiró la idea de examinar si la hipoxia podría aumentar la longevidad y alargar el periodo reproductivo de los rotíferos en nuestros estudios sobre la biología del envejecimiento. En este trabajo mostramos que la exposición de rotíferos a cuatro días en una atmósfera de 1.6 % de* O_2 *alargó la vida media en un 107 % frente los controles en normoxia (20.9 % de* O_2*), cuya vida media era de 7.9 días. La alternancia de días de hipoxia y de normoxia también aumentó marcadamente la vida media de los rotíferos, en un 53 %. La exposición a la hipoxia (1.6 % de* O_2*) casi duplicó la vida media reproductiva de las hembras (24.3 vs 12.4 descendientes en el control). La exposición a la hipoxia protegió a los rotíferos del subsecuente estrés oxidativo y del ocasionado por la radiación ultravioleta, pero no del estrés debido a la inanición, la adversidad osmótica o térmica. Las clases de edad de 0-4 días respondieron mejor a la exposición a la hipoxia en comparación con la exposición a la hipoxia de las de 4-8 u 8-12 días. Los efectos protectores de dos días de exposición a la hipoxia persistieron hasta el día 6 aunque desaparecieron el día 8. Los huevos de diapausa de rotíferos son especialmente resistentes a la hipoxia y eclosionan en atmósferas con niveles tan bajos como 1.6 % de* O_2 *tan sólo con un día de retraso. Nuestra conclusión es que la exposición a la hipoxia durante cuatro días en las clases de edad más jóvenes aumenta la longevidad del rotífero y alarga la etapa reproductiva. Esta respuesta tiene un valor adaptativo en los sedimentos anóxicos en los que se depositan la mayoría de los huevos de diapausa de los rotíferos.*

Palabras clave: *hipoxia, rotíferos, Brachionus, vida media*

INTRODUCTION

It is difficult to predict where inspiration for scientific investigations will originate. Moreover, there often is a long lag time between publication of a paper and incorporating its findings into research. An example is a paper that one of us read with keen interest in 1989 by Esparcia, Miracle and Serra describing the adaptation of natural populations of *Brachionus plicatilis* to low oxygen environments. This species apparently is able to slow its metabolism in hypoxia, altering its reproductive schedule and extending generation times. These authors suggested that this is accomplished by switching to fermentative metabolic pathways, reducing their respiratory oxygen demand. This adaptation allowed *B. plicatilis* to occupy low oxygen environments in oxyclines of meromictic lakes that were not habitable by other zooplankters. While fascinating, this paper did not immediately indicate how to incorporate this result into our research program.

More than 20 years later, our lab group was investigating the biology of aging using the rotifer *B. plicatilis* as an experimental model. There has been a resurgence of interest in rotifers as models of aging (Snell, 2014; Snell *et al.*, 2015; Kaneko *et al.*, 2017; Gribble & Snell, 2018) and we were keen to find means to extend lifespan and healthspan with minimal environmental interventions. We stumbled across an interesting result of lifespan extension by using glycerol as a dietary supplement (Snell & Johnston, 2014). Observations led us to hypothesize that glycerol supplements caused rotifers to shift their metabolism away from glycolysis toward oxidative phosphorylation. We recalled the metabolic shift associated with hypoxia hypothesized by Esparcia *et al.* (1989) and wondered whether exposing rotifers to hypoxia might also extend their lifespan. This idea caused us to embark on a series of experiments, the results of which are described in this paper. We examined how hypoxia modifies longevity and reproduction in the rotifer *Brachionus manjavacas*, how hypoxia treatments protect rotifers from subsequent oxidative and UV stress, what age-classes best respond to hypoxia exposure, and how long the protective effects of hypoxia persist. We also demonstrate that rotifer diapausing eggs are especially resistant to hypoxia and hatch in atmospheres containing as little as 1.6 % O_2.

METHODS

Rotifer Species and Culturing

We used *Brachionus manjavacas* (Fontaneto *et al.*, 2007) as experimental animals in this study. *B. manjavacas* was originally collected from Azov Sea and previously known as *Brachionus*

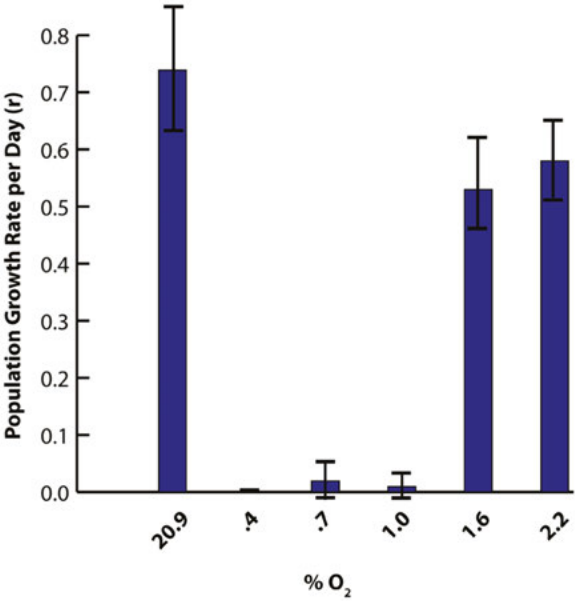

Figure 1. Three-day reproductive assays with varying concentrations of oxygen. *B. manjavacas* neonates were exposed to 0.4, 0.7, 1.0, 1.6, or 2.2 % O_2 for 3 days. N = 24 for all five treatments and the control. Percentages represent % of atmospheric O_2 concentration in treatments. The population growth rates (r, offspring per female per day) for each treatment are plotted. Vertical lines indicate standard errors. There were significant decreases in reproduction at 0.4, 0.7 and 1.0 % O_2. Population growth rates at O_2 concentrations higher than 1.6 % were not significantly different from control. *Ensayos de reproducción de tres días variando la concentración de oxígeno. Los neonatos de* B. manjavacas *fueron sometidos a valores de 0.4, 0.7, 1.0, 1.6 y 2.2 %* O_2 *durante los tres días. N = 24 para los cinco tratamientos y el control. Los porcentajes representan el % de la concentración de* O_2 *atmosférico en los tratamientos. Se representa la tasa de crecimiento de la población para cada tratamiento (r, descendencia por hembra y día). Las líneas verticales representan el error típico. Hubo descensos significativos en la reproducción a 0.4, 0.7 y 1.0 % de* O_2*. Las tasas de crecimiento poblacional a concentraciones de* O_2 *mayores del 1.6 % no fueron significativamente diferentes del control.*

plicatilis (Snell *et al.*, 2006; Fontaneto *et al.*, 2007). *B. manjavacas* has been cultured continuously in our laboratory since 1983 (Stout *et al.*, 2010). Rotifers were cultured in 15 ppt artificial seawater (ASW, prepared from Instant Ocean salts) exposed to constant fluorescent illumination (2000 lux) at 25 °C in all of the following experiments, except if otherwise specified. *B. manjavacas* is a cyclical parthenogen whose life cycle is well understood (Wallace & Snell 2010).

Oxygen Conditions

B. manjavacas diapausing eggs were hatched in normoxic culture medium in an atmosphere of 20.9 % O_2. Hatchlings, which are asexual females, were transferred to the oxygen treatments within 2 hours of hatching. Desired oxygen concentrations were achieved by flushing a Coy Lab Products anaerobic chamber (approximately 1 m^3 volume) with N_2 until the desired O_2 atmospheric concentrations were reached. This chamber had an oxygen controller, which measured O_2 concentrations in air. Because our experimental units were 24-well plates with 1 ml in each well, we assumed that O_2 concentration in the medium rapidly came into equilibrium with the O_2 concentration in the atmosphere. The small experimental volumes prevented direct measurement of O_2 concentration in water using an O_2 electrode. The rapidly detectable biological responses in our O_2 treatments support this interpretation.

3-Day Reproductive Assays

Neonates were immediately exposed to 20.9 %, 0.1 %, 0.4 %, 0.7 %, 1.0 %, 1.6 % or 2.2 % O_2 atmospheres and fed 2 x 10^5 *Tetraselmis suecica* cells/ml for 3 days. We recorded the total offspring produced via asexual reproduction during the 3-day period. From these data, instantaneous population growth rate per female per day, r, was calculated as:

$$r = (\ln Nt - \ln N_0)/T$$

where: Nt = number of rotifers after 3 days, N_0 = initial number of rotifers per well (1), and T = 3 days.

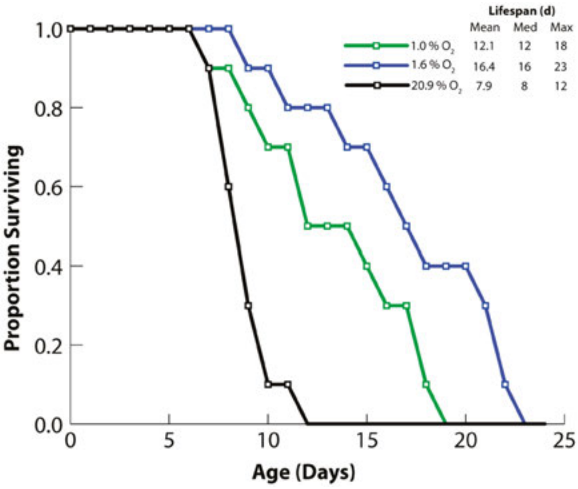

Figure 2. Age-specific survival of *B. manjavacas* exposed to hypoxic conditions. *B. manjavacas* neonates were continuously exposed to 1.0 % (green), 1.6 % (blue) or 20.9 % (black) O_2 over their lifespan. The mean, median and maximum lifespan for each treatment are reported in days. The proportion surviving represents the portion of the initial cohort of 120 females surviving to the indicated age. Treatment with 1.6 % O_2 resulted in a significant increase in the lifespan of the females. *Supervivencia por edad de* B. manjavacas *expuesta a condiciones de hipoxia. Neonatos de* B. manjavacas *fueron expuestos continuamente a 1.0 % (verde), 1.6 % (azul) o 20.9 % (negro)* O_2 *a lo largo de su vida. La media, mediana y máxima esperanza de vida para cada tratamiento esta expresada en días. La proporción de sobrevivientes representa la porción de la cohorte inicial de 120 hembras que sobreviven a la edad indicada. Tratamiento con 1.6 %* O_2 *resultó en un incremento den la esperanza de vida de las hembras.*

Life Table Experiments

Full cohort life tables were performed with 24 female rotifers per treatment. Animals were kept in 24-well plates with 1 female per well in 1 ml medium containing 2 x 10^5 *T. suecica* cells/ml. Plates were maintained at 22 °C in the dark in either normoxic conditions or continuous or daily alternating hypoxic conditions of 1.6 % O_2. Offspring were counted and removed daily. The original parthenogenetic mothers were transferred to fresh plates on day 6. Plates were monitored daily for mortality until all animals were dead. Data are reported as mean, median and maximum lifespan (age of 95 % mortality).

Stressor Challenge Experiments

Rotifers neonates were exposed to 1.6 % O_2 for 2 days and fed 6 x 10^5 *T. suecica* cells/ml with 20 µM 5-fluoro-2’-deoxyuridine (FDU) (Snell *et al.*, 2012) at 25 °C. FDU prevents asexual eggs from hatching, eliminating the challenge of discriminating maternal females from F1 offspring. This simplifies experiments and reduces experimental errors. The algae was removed from the medium by filtration. Rotifers were then exposed to stressors, and transferred to fresh ASW for 72-hour recovery, and survival was recorded. The following stressors were applied: (1) 72 h starvation, (2) 20 min UV-B exposure, (3) 72 h oxidative stress, (4) 1 h osmotic shock, and (5) 1 h heat shock. UV stress was accomplished by exposing the animals to UV-B radiation for 20 min, 25 cm from an 8 W source (UVP, model UVM-28 EI) with an intensity peak at 302 nm. Rotifers were exposed in 5 ml ASW in a 55 mm diameter petri dish so that they received a dose of about 130 J/m^2. Oxidative stress was accomplished by exposing the animals to 0.1 µM juglone for 72 hours. Transferring animals from 15 ppt ASW to 60 ppt ASW for 1 hour produced osmotic stress. They were transferred back to 15 ppt ASW and followed for 72 hours. Finally, heat shock was accomplished by transferring the animals from 22 °C to 40 °C for 1 hour. The animals were transferred to 24 well plates at 25 °C with 1 ml 15 ppt ASW, 20 µM FDU, and no algae to follow post-stress survival for 72 hours. Each stressor challenge experiment was completed with 12 replicates per treatment, each containing 10 animals.

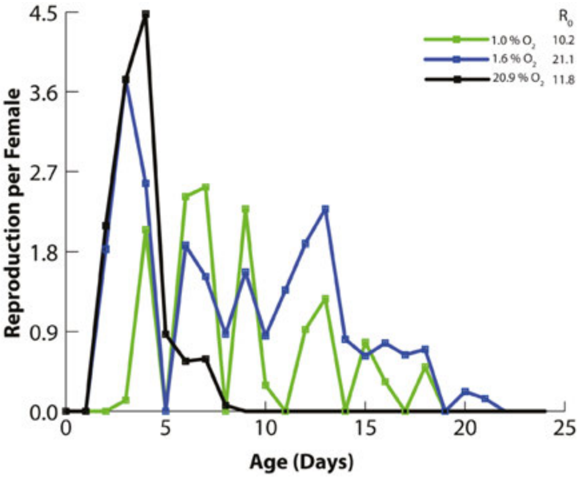

Figure 3. Age-specific reproduction of *B. manjavacas* exposed to hypoxic conditions. *B. manjavacas* neonates were continuously exposed to 1.0 % (green), 1.6 % (blue) or 20.9 % (black) O_2 over their lifespan. Reproduction per female refers to the average daily reproduction by a cohort of 120 females. R_0 is the mean total reproduction of a female over her lifetime. *B. manjavacas* neonates were continuously exposed to either 1.0 % (green), 1.6 % (blue) or 20.9 % (black) O_2 over their lifespan resulted in a significant increase in lifetime reproduction (R_0) of the females. *Edad específica de reproducción de* B. manjavacas *expuesta a condiciones hipóxicas. Neonatos de* B. manjavacas *se expusieron en continuo a 1.0 % de O_2 (verde), 1.6 % de O_2 (azul), o 20.9% de O_2 (negro) a lo largo de su vida. La reproducción por hembra se refiere a la reproducción diaria promedio de una cohorte de 120 hembras. R_0 es la reproducción media total de una hembra a lo largo de su vida. La exposición continua de neonatos de* B. manjavacas *a 1.0 % (verde) y 1.6 % (azul) o a 20.9 % (negro) de O_2 a lo largo de su vida resultó en un incremento significativo en la reproducción de las hembras durante el tiempo de vida (R_0).*

Retention of Stress Protection

Retention of oxidative stress protection was examined by exposing neonates to 1.6 % O_2 for 2 days, whereas control animals remained at 20.9 % O_2. The hypoxic treated animals were then transferred to normoxic conditions where they were incubated at 25 °C until removed on days 4, 6 or 8 to complete oxidative stress tests by exposing the animals to 0.1 µM juglone for 72 hours.

Effect of Age of Hypoxia Exposure on Lifespan Extension

Treating rotifers with hypoxia at different ages tested whether age of exposure alters the protective effects of hypoxia. Animals were transferred from normoxic conditions to 1.6 % O_2 for 0–4 days, 4-8 days or 8-12 days after hatching. The animals then were transferred back to normoxic conditions and followed until death. Full cohort life tables were performed with 120 female rotifers per treatment. Animals were kept in 24-well plates with 5 females per well in 1 ml medium containing 6 x 10^5 *T. suecica* cells/ml in 15 ppt ASW with 20 µM FDU. Plates were maintained at 22 °C in the dark. All maternal females were transferred to fresh plates on day 6. Plates were monitored daily for mortality until all animals were dead. Data are reported as mean, median and maximum lifespan.

Diapausing Egg Hatching in Hypoxia

Four replicate 1 ml wells of 15 ppt ASW containing 100 diapausing eggs were exposed to 20.9 % O_2 or 1.6 % O_2 at 22 °C in fluorescent light of 2000 lux. The number hatching was recorded at 24, 48 and 72 h.

Statistics

Reproductive output and stress challenge tests were analyzed using an ANOVA with Dunnett's test comparing treatments to control. Life table experiments were analyzed by using the JMP Pro 12 (SAS Institute) reliability and survival analysis with Wilcoxon's test to compare survival curves.

RESULTS

We screened a range of oxygen concentrations (0.4 % - 20.9 %) for their effect on *B. manjavacas* reproduction using a 3-day reproductive test (Fig. 1). The treatments 1.6 % and 2.2 % O_2 produced r-values of 0.53 and 0.57 per day, which were not significantly different from the control (20.9 % O_2) with an r-value of 0.74. In contrast, exposure to O_2 concentrations 0.4 %, 0.7 % or 1.0 %,

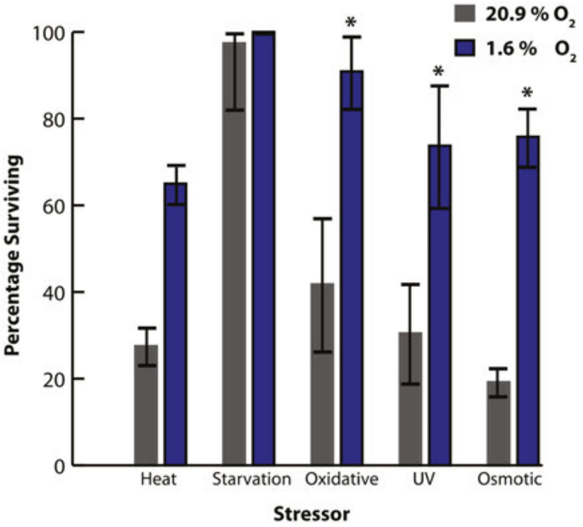

Figure 4. Effect of stressor challenges on *B. manjavacas* survival. *B. manjavacas* neonates were exposed to either 20.9 % (control) or 1.6 % O_2 (hypoxia) for 2 days, then subjected to the following stressors: starvation, heat, oxidation, osmotic stress, or UV irradiation. Percentages represent % of atmospheric O_2 concentration in treatments. Proportion surviving is the fraction of a cohort of 120 rotifers surviving 48 hours after stressor exposure. Vertical lines indicate standard error. Asterisks indicate significant differences from control by ANOVA and Dunnet's test. *Efecto de los desafíos estresores sobre la supervivencia de* B. manjavacas. *Los neonatos fueron expuestos a 20.9 % de O_2 (control) o 1.6 % O_2 (hipoxia) por 2 días, y entonces fueron sometidos a los siguientes estresores: inanición, calor, oxidación, estrés osmótico o radiación UV. Los porcentajes representan el % de la concentración de O_2 atmosférico en los tratamientos. La proporción superviviente es la fracción de una cohorte de 120 rotíferos que sobreviven 48 horas después de la exposición al estresor. Las líneas verticales indican error estándar. Los asteriscos indican diferencias significativas respecto al control mediante ANOVA y el test de Dunnet.*

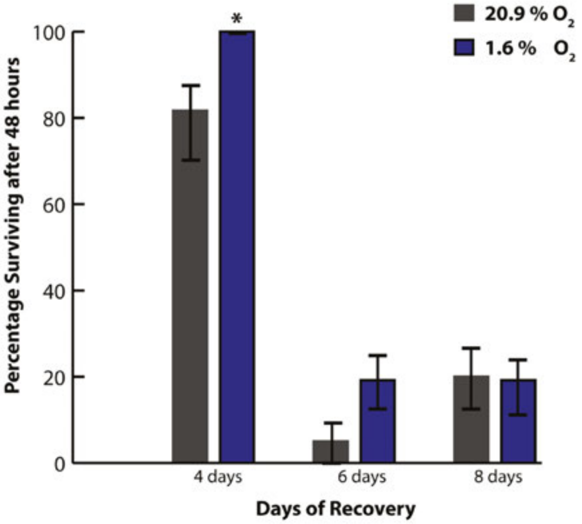

Figure 5. Loss of hypoxia protection. *B. manjavacas* neonates were exposed to either 20.9 % (control) or 1.6 % O_2 (hypoxia) for 2 days. Percentages represent % of atmospheric O_2 concentration in treatments. The females were then transferred to 20.9 % O_2 and tested for resistance to oxidative stress (0.1 µM juglone) on days 4, 6, or 8 of their lifespan. Hypoxia exposure provided protection from oxidative stress through day 6, the protection was lost by day 8. Vertical lines indicate standard errors and asterisks show significant differences from control by ANOVA and Dunnet's test. *Pérdida de la protección de la hipoxia. Neonatos de* B. manjavacas *se expusieron a 20.9 % de O_2 (control) o 1.6 % de O_2 (hipoxia) durante dos días. Las hembras fueron entonces transferidas al 20.9 % de O_2 y probadas para su resistencia al estrés oxidativo (0.1 µM juglone) los días 4, 6 u 8 de su vida. La exposición a la hipoxia proporcionó protección contra el estrés oxidativo hasta el día 6, la protección se perdió el día 8. Las líneas verticales indican los errores estándar y los asteriscos muestran diferencias significativas frente al control mediante ANOVA y la prueba de Dunnet.*

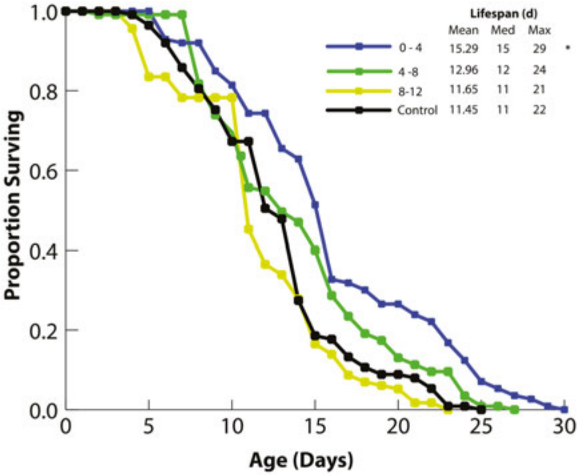

Figure 6. Effect of age of hypoxia exposure on lifespan extension. *B. manjavacas* neonates were exposed to 1.6 % O_2 for different periods throughout their lifespan, either days 0–4 (red), days 4–8 (green) or days 8–12 (blue). Control was continuously exposed to 20.9 % O_2. Percentages represent % of atmospheric O_2 concentration in treatments. The rotifers were treated at normoxic (20.9 %) O_2 during the periods before and after hypoxia treatment. The mean, median and maximum lifespan for each treatment are reported in days. The proportion surviving represents the portion of the initial cohort of 120 females surviving to the indicated age. Only treatment from day 0–4 resulted in a significant increase in their lifespan by Wilcoxson's test of survival curves. *Efecto de la edad de la exposición a la hipoxia en el aumento de la esperanza de vida. Neonatos de* B. manjavacas *fueron expuestos a 1.6 % de O_2 en diferentes períodos a lo largo de su vida, los de 0-4 días (rojo), los de 4-8 días (verde) o los de 8-12 días (azul). El control fue continuamente expuesto a 20.9 % de O_2. Los porcentajes representan el % de la concentración de O_2 atmosférico en los tratamientos. Los rotíferos fueron tratados con normoxicidad (20.9 %) de O_2 durante los períodos antes y después del tratamiento de hipoxia. La vida media, mediana y máxima para cada tratamiento se expresaron en días. La proporción de supervivientes representa la porción de la cohorte inicial de 120 hembras que sobreviven a la edad indicada. Solo los del tratamiento de 0-4 días dieron como resultado un aumento significativo en su esperanza de vida.*

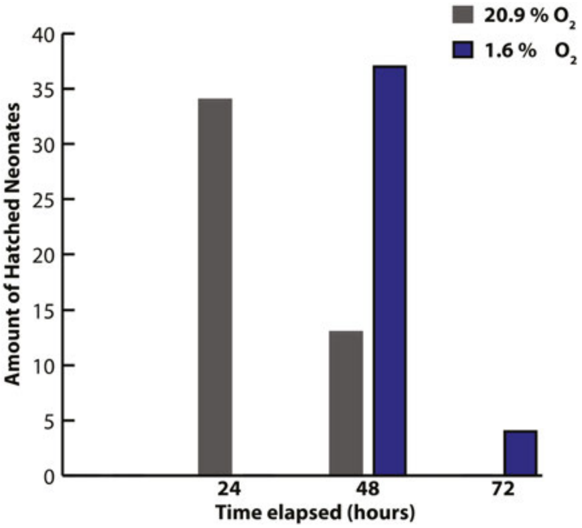

Figure 7. Diapause egg hatching is only delayed in hypoxia. *B. manjavacas* diapausing eggs were hatched in either 20.9 % or 1.6 % O_2. Percentages represent % of atmospheric O_2 concentration in treatments. The total number of females hatched was not significantly different, however there was a 24 h delay in hatching at 1.6 % O_2. Vertical lines indicate standard errors. *La eclosión del huevo de diapausa solo se retrasa con la hipoxia. Los huevos de diapausa de* B. manjavacas *se incubaron en 20.9 % o 1.6 % de O_2. Los porcentajes representan el % de la concentración de O_2 atmosférico en los tratamientos. El número total de hembras nacidas no fue significativamente diferente; sin embargo, hubo un retraso de 24 h en la eclosión de los incubados con el 1.6 % de O_2. Las líneas verticales indican los errores estándar.*

resulted in at least a 10-fold decrease in reproductive rate compared to the control in normoxia.

We exposed rotifer females in a cohort life table experiment continuously to 20.9 % or 1.6 % O_2 concentrations, or alternating days of normoxia and 1.6 % hypoxia (Fig. 2). Exposure to continuous 1.6 % O_2 resulted in a 107 % increase in mean lifespan from 7.9 days in control to 16.4 days in hypoxia. This result was significant with $p < 0.001$ by Wilcoxson's test. Mean lifespan likewise significantly increased in the alternating day 1.6 % O_2 treatment by 53 % to 12.1 days.

Mean lifetime reproduction (R_o) of rotifers exposed to 1.6 % O_2 was nearly double that of the control (24.3 vs 12.4 offspring) (Fig. 3). This effect was achieved primarily by higher reproduction in older age classes compared to control. In contrast, there was no significant difference between the alternating day 1.6 % O_2 treatment (13.5 offspring) and the control.

Animals exposed to 1.6 % O_2 for two days exhibited greater survival than the controls exposed to oxidative or UV stress, but not those exposed to heat, osmotic, or starvation stress (Fig. 4). Pre-treatment of rotifers for two days with 1.6 % hypoxia increased their survival of oxidative stress by 2.7-fold. Similar hypoxia exposure improved rotifer resistance to UV exposure by

4.2-fold. The protective effect of hypoxia was only temporary (Fig. 5), persisting until day 6, but after 8 days the hypoxia protection vanished.

We investigated in which age classes hypoxia exposure provided the greatest lifespan extension. Rotifers were exposed to 1.6 % O_2 hypoxia for specific time periods: ages 0-4, 4-8, or 8-12 days (Fig. 6). For periods not exposed to hypoxia, animals were in 20.9 % O_2 normoxia. Only hypoxia treatment from ages 0-4 days resulted in a significant increase in rotifer lifespan.

Exposure of diapausing eggs to hypoxia during hatching delayed their hatching by 24 h (Fig. 7). Although delayed, a similar fraction of diapausing egg eggs hatched by 72 h (40 vs 48 % in control).

DISCUSSION

Our principal findings are that *B. manjavacas* tolerate severe hypoxia of 1.6 % O_2 which is 12X lower than normal atmospheric O_2. Hypoxia exposure extended rotifer lifespan and enhanced lifetime reproduction, primarily by increasing reproduction in older age classes. Pre-exposure of rotifers to hypoxia conferred protection from subsequent oxidative and UV stress, however benefits of hypoxia exposure vanished after 8 d. The benefits of hypoxia exposure were only realized if exposure included the youngest age classes. Rotifer diapausing eggs can hatch in 1.6 % hypoxia just as well as in normoxia, but are delayed by 24 h.

The first report of high tolerance of hypoxia by species in the *B. plicatilis* species complex was by Esparcia *et al.* (1989). They found strong rotifer reproduction ($r = 0.7\ d^{-1}$) and the maintenance of high population densities (70 rotifers ml^{-1}) at O_2 concentrations as low as 1 mg/L. Seawater saturates with about 7 mg O_2/L at 25 °C, so 1 mg/L is about 14 % saturation. This compares with our results with *B. manjavacas* that identified an O_2 concentration threshold for vigorous reproduction at about 8 % saturation. Esparcia *et al.* confirmed that when O_2 concentration became anoxic, their population rapidly crashed to extinction, similar to our findings for *B. manjavacas*. Esparcia *et al.* also reported that *B. plicatilis* tolerated hypoxia by lowering its metabolic rate. Hypoxia is a regular feature of many natural rotifer environments, including seasonal oxyclines where surface waters are more oxygenated than the hypolimnion (Miracle & Vicente, 1983). Since *B. manjavacas* is adapted to low O_2 environments on the edge of anoxic zones, they are pre-adapted to tolerating hypoxia in high-density cultures. This makes them valuable as a larval fish food in aquaculture (Hagiwara *et al.*, 2017).

Hypoxia inducible factor (HIF) proteins have a central role in responding to changes in environmental oxygen (Jiang *et al.*, 2001). HIF proteins are transcription factors stabilized by low-oxygen, modulating the expression of hundreds of genes to produce the hypoxic response (Hochachka & Lutz, 2001; Ke & Costa, 2006). Induction of HIF-1 expression in the nematode *C. elegans* increased longevity and healthspan (Zhang *et al.*, 2009; Leiser *et al.*, 2015). So, it is not surprising that exposure to hypoxic conditions had a lifespan extending effect on *B. manjavacas*.

Ozaki *et al.* (2010) reported that caloric restriction is prevalent at high population densities in stationary phase of *B. plicatilis* cultures, shifting metabolism from aerobic to anaerobic pathways. These authors speculated that this shift is the mechanism that provides hypoxia tolerance, similar to what was hypothesized by Esparcia *et al.* (1992).

We conclude that early studies by Maria Rosa Miracle's group demonstrated that brachionid rotifers adapted to hypoxic environments in meromictic lakes. This work foreshadowed how these traits could be exploited in high-density rotifer cultures in marine finfish larviculture and pointed to how hypoxia might be manipulated to extend lifespan and healthspan in rotifer studies on the biology of aging. Like so many of her studies, Maria Rosa's investigation of hypoxia led to much broader influence on rotifer research.

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Zooplankton distribution, diversity and taxonomy

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An account on the non-malacostracan crustacean fauna from the inland waters of Crete, Greece, with the synonymization of *Arctodiaptomus piliger* Brehm, 1955 with *Arctodiaptomus alpinus* (Imhof, 1885) (Copepoda: Calanoida)

Federico Marrone[1,*], Giuseppe Alfonso[2], Fabio Stoch[3], Valentina Pieri[4], Miguel Alonso[5], Michalis Dretakis[6] and Luigi Naselli-Flores[7]

[1] Department of Biological, Chemical and Pharmaceutical Sciences and Technologies – Section of Zoology, University of Palermo, via Archirafi 18, 90123 Palermo, Italy.
[2] Di.S.Te.B.A., University of Salento, s.p. Lecce-Monteroni, 73100 Lecce, Italy.
[3] Evolutionary Biology & Ecology, Université Libre de Bruxelles, Avenue F.D. Roosevelt 50, 1050 Brussels, Belgium.
[4] University of Parma, Department of Chemistry, Life Science and Environmental Sustainability, 43124 Parma, Italy.
[5] Ecology Section, Department of Evolutionary Biology, Ecology and Environmental Sciences, Faculty of Biology, University of Barcelona, Avda. Diagonal 643, 08028 Barcelona, Spain.
[6] Natural History Museum of Crete, University of Crete, Knossos Av., P.O. Box 2208, 714 09 Heraklion, Crete, Greece.
[7] Department of Biological, Chemical and Pharmaceutical Sciences and Technologies – Section of Botany and Plant Ecology, University of Palermo, via Archirafi 28, 90123 Palermo, Italy.

[*] Corresponding author: federico.marrone@unipa.it

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ABSTRACT

An account on the non-malacostracan crustacean fauna from the inland waters of Crete, Greece, with the synonymization of *Arctodiaptomus piliger* Brehm, 1955 with *Arctodiaptomus alpinus* (Imhof, 1885)

The Mediterranean bioregion is widely recognised as a biodiversity hotspot and its inland waters are among the species richest ecosystems of the northern hemisphere. However, the extent of such biodiversity has not been totally unravelled, especially in the Mediterranean islands. Here we present a first account of the crustaceans inhabiting 21 permanent and temporary ponds in Crete, the largest of the Greek islands and the fifth largest island in the Mediterranean Sea. The ponds, sampled between 2009 and 2018, cover all the island surface even though their number cannot be considered exhaustive to represent the entire non-malacostracan fauna of the island. Nevertheless, 46 taxa were identified and most of them are new records for Crete. Moreover, molecular taxonomy allowed to solve the systematic position of *Arctodiaptomus piliger* Brehm, 1955 and to synonymize this organism, previously considered endemic of the island, with *Arctodiaptomus alpinus* (Imhof, 1885). As regard branchiopods, this paper contributes a step ahead to clarify the taxonomic position of the Mediterranean *Chirocephalus* and *Ceriodaphnia* species. Finally, a review of all the non-stygobitic species present on the island is reported, including 78 taxa (21 Branchiopoda, 28 Copepoda and 29 Ostracoda). Overall, the achieved results offer new clues to solve the complex biogeographical pattern of the "entomostracan" crustaceans inhabiting the inland waters of the Mediterranean region.

Key words: Mediterranean biodiversity, temporary ponds, Calanoida, Branchiopoda, Ostracoda

RESUMEN

Listado de la fauna de crustáceos no malacostráceos de las aguas epicontinentales de Creta, Grecia, y sinonimización de

Arctodiaptomus piliger ***Brehm, 1955 con*** **Arctodiaptomus alpinus** ***(Imhof, 1885) (Copepoda, Calanoida)***

La bioregion mediterránea es ampliamente reconocida como un hotspot de biodiversidad, y sus aguas epicontinentales se encuentran entre los ecosistemas más ricos en especies del hemisferio septentrional. Sin embargo, el alcance de dicha biodiversidad aún no ha podido ser totalmente evaluado, especialmente en las islas mediterráneas. En el presente artículo se presenta una primera relación de los crustáceos no malacostráceos que habitan en 21 lagunas permanentes y temporales de Creta, la mayor de las islas griegas y la quinta más grande del mar Mediterráneo. Las lagunas, muestreadas entre 2009 y 2018, se encuentran distribuidas por toda la superficie de la isla aunque su número no puede considerarse lo suficientemente exahustivo como para representar a la totalidad de la fauna de crustáceos no malacostráceos de la isla. No obstante, se identificaron 46 taxones, la mayor parte de ellos nuevas citas para Creta. Además, la taxonomía molecular permitió solventar la posición taxonómica de Arctodiaptomus piliger *Brehm, 1955 y sinonimizar este organismo, previamente considerado endémico de la isla, con* Arctodiaptomus alpinus *(Imhof, 1885). En lo referente a los branquiópodos, el presente artículo constituye un avance significativo para clarificar la posición taxonómica de las especies mediterráneas de* Chirocephalus *y* Ceriodaphnia*. Finalmente, se reporta una revisión de todas las especies no hipogeas de la isla que incluye 78 taxones (21 Branquiópodos, 28 copépodos y 29 ostrácodos). En conjunto, los resultados obtenidos ofrecen nuevas pistas para resolver el complejo patrón biogeográfico de los crustáceos "entomostráceos" que viven en las aguas epicontinentales de la region mediterránea.*

Palabras clave: *Biodiversidad mediterránea, lagunas temporales, Calanoida, Branchiopoda, Ostracoda*

INTRODUCTION

Biodiversity in the Mediterranean Basin is amongst the highest recorded in any biogeographic region, both in terms of species richness and endemism (Blondel *et al.*, 2010). This high diversity largely depends on the remarkable variability of the landscape and results from a complex combination of climatic, geological and paleogeographic factors which make the Mediterranean Basin a "hotspot" of biodiversity: i.e. a varied and fragmented mosaic of ecosystems and communities (Allen, 2001).

Some components of this Mediterranean environmental mosaic (e.g. terrestrial and marine flora, and vertebrate fauna) have been intensively studied in the past decades. However, natural freshwater ecosystems remain relatively unexplored habitat, especially as regard their "microscopic" biota. Although several man-made lakes are scattered all around the Mediterranean area, small water bodies and wetlands are undoubtedly the most characteristic aquatic ecosystems in the region (Alvarez Cobelas *et al.*, 2005). Several thousands of small-sized water bodies (area < 0.001 km^2) are located in Southern Europe (Meybeck, 1995) and they largely contribute to enhance regional biodiversity by hosting a number of species significantly higher than that present in large lakes (Downing, 2010), making Mediterranean small water bodies themselves "hotspots within hotspots" of biodiversity. Moreover, freshwaters are among the most threatened ecosystems in the world because of anthropic pressure, i.e. industrialization of agriculture processes and urbanization (Dudgeon *et al.*, 2006). Actually, since the importance of small water bodies has suffered a lack of scientific recognition in the second half of the past century, several wetlands and ponds have been drained and reclaimed. These actions have contributed to the rarefaction, at a very fast rate, of these freshwater ecosystems and their biota in all the Mediterranean Region (Zacharias & Zampas, 2010; Stoch *et al.*, 2016). The disappearing of freshwater ecosystems, coupled with the hydrological variability caused by climate change, the over-exploitation of water resources, and the changes induced in natural communities by the introduction of exotic species, highlights the urgency of biodiversity assessments in all the Mediterranean inland waters (Moreno *et al.*, 2017). This is particularly true as regard the passively dispersed fauna inhabiting the inland waters of the Mediterranean islands and archipelagos. Actually, the success of dispersal for these species greatly depends on the number of ponds scattered across a given territory (Incagnone *et al.*, 2015); the aquatic ecosystems on the islands acting as stepping-stones in the migration corridors of birds which transport resting stages and propagules across the Mediterranean Sea (Hindmarch & Kirby, 2002). Therefore, getting a knowledge as much as possible complete of the biodiversity

Figure 1. Location of the sampled sites. White circles: temporary waterbodies; black circles: permanent waterbodies. See table 1 for their names, codes, and coordinates. *Situación de las localidades muestreadas. Círculos blancos: masas de agua temporales; círculos negros: lagos permanentes. En la tabla 1 se indican sus nombres, códigos y coordenadas.*

of aquatic organisms in insular inland waters is of paramount importance to have a thorough vision on the biogeography of aquatic organisms in the Mediterranean region, and to develop sound conservation plans for these characteristic ecosystems (e.g. Alfonso *et al.*, 2011; Bagella *et al.*, 2016; Diaz-Paniagua, 2015; Grillas *et al.*, 2004).

The crustacean fauna of aquatic ecosystems in the islands of the western Mediterranean Basin has been sufficiently documented, and several investigations were carried out in the last decades addressed toward the faunistic exploration of Sicily (Marrone & Mura, 2006; Marrone *et al.*, 2005, 2006a, 2006b; Pieri *et al.*, 2006), Maltese islands (Lanfranco, 2001), Sardinia (Ruffo & Stoch, 2005; Boix *et al.*, 2017, and references therein), Corsica (Champeau & Thiéry, 1990; Culioli *et al.*, 2006) and Balearic Islands (Jaume, 1989-1990; Pretus, 1990; Zamora *et al.*, 2005). Conversely, with the exception of Corfu (Stephanides, 1948), only a few, scattered data are available for the islands located in the eastern Mediterranean Sea (e.g. Mura & Hadjstephanou, 1987; Tziortzis *et al.*, 2014; Karagianni *et al.*, 2018); remarkably, the availability of data for the Aegean Islands is very scarce.

In this paper, we provide a first faunal assessment of the non-malacostracan crustaceans inhabiting permanent and temporary ponds in Crete, the southernmost island of the Aegean archipelago and the fifth Mediterranean island by surface, with the aim to increase our knowledge on the biogeography of freshwater crustaceans passively dispersing across the Mediterranean Basin. To our knowledge, despite Crete has the highest amount of Mediterranean Temporary Ponds (priority habitat 3170* according to the EU "Habitats Directive") among the administrative regions of Greece (Dimitriou *et al.*, 2006), just a few papers dealing with Cretan inland water crustaceans are available in the scientific literature (see Kiefer, 1928; Chappuis, 1929, 1956; Klie, 1941; Brehm, 1955; Lindberg, 1956; Pesce & Maggi, 1981; Petkowski *et al.*, 2000). Moreover, with this paper we wish to commemorate the late Prof. Maria Rosa Miracle, who recently passed away after an illness of over one year. With her work, Maria Rosa largely contributed to establish a Mediterranean vision of Limnology and promoted the importance of Mediterranean small water bodies among dozens of students and colleagues.

MATERIAL AND METHODS

Crete, the largest island of Greece and the fifth in the Mediterranean Sea, is a limestone island covering an area of 8303 km^2. It is located about 100 km south of the Greek mainland, and 175 km south-west of Turkey, along a line crossing the

Table 1. List of the sampled sites. Geographical coordinates are expressed as decimal degrees (Map Datum: WGS84). GSD: "Greek Samples Database"; Hydr.: Hydroperiod; P: permanent; T: temporary. *Relación de las localidades muestreadas. Las coordenadas geográficas se expresan en grados decimales (Datum: WGS84). GSD: "Greek Samples Database"; Hydr.: Hidroperiodo; P: permanente; T: temporal.*

GSD Code	Site	Latitudine N	Longitudine E	Elevation (m a.s.l.)	Habitat type	Hydr.	Plankton sample	Sediment sample
GR082	Limni Kourna	35.329935	24.272689	16	Lake	P	x	
GR083	Limni Agya	35.476936	23.932054	37	Reservoir	P	x	
GR084	Pond 1 at Omalos (Chania)	35.324877	23.890936	1060	Pond	T	x	x
GR085	Pond along the Exo-Apidi road	35.068422	26.124883	605	Reservoir	P	x	
GR086	Marsh 1 of Aposelemis	35.334893	25.328806	0	Ground pools	T	x	
GR087	Limni Zirou	35.072129	26.137817	566	Pond	P	x	
GR088	Pond 2 at Omalos (Chania)	35.326636	23.888916	1054	Pond	T	x	x
GR090	Marsh of Xerocampos	35.047924	26.237118	0	Marsh	T		x
GR091	Marsh 2 of Aposelemis	35.335044	25.327582	0	Marsh	T		x
GR092	Marsh of Vai	35.255259	26.264846	0	Marsh	T		x
GR094	Pond 3 at Omalos (Chania)	35.325999	23.890255	1057	Pond	T		x
GR095	Faistos rock pool	35.051189	24.814912	76	Rock pool	T		x
GR096	Spinalonga rock pool	35.299204	25.738825	10	Rock pool	T		x
GR097	Limni Katalyki	35.075107	24.745754	0	Pond	T		x
GR098	Athoudiaris pool	35.235600	26.224500	558	Mud puddle	T		x
GR099	Karst pool	35.051979	26.186606	539	Pond	T		x
GR100	Pond at Omalos (Dykti)	35.071527	25.454529	1330	Pond	T	x	x
GR151	Stephana pond	35.264155	24.893111	1128	Mud puddle	T	x	
GR152	Karydi pool	35.130936	26.164500	685	Pond	T	x	
GR153	Profitis Ilias pool	35.227659	26.219754	199	Mud puddle	T		x
GR175	Cape Lithinon rock pool	34.945553	24.761992	152	Rock pool	T	x	

large islands of Karpathos and Rhodes. Cretan landscape is dominated by mountain ranges, with the highest peak reaching 2456 m a.s.l. and a mean elevation of 482 m a.s.l. The climate is typically Mediterranean at the low altitudes, but on the mountains snow coverage can easily persist until May, with bioclimatic belts ranging from the Thermomediterranean to the Supramediterranean one (Rivas-Martínez *et al.*, 2004).

Crustacean samples were collected throughout the island from November 2009 to January 2018; the geographical coordinates and elevation of each site were recorded using a GPS, and an alphanumeric code referring to a "Greek Samples Database" (GSD) set up by FM and FS, was attributed to each of them (Fig. 1; Table 1). When water bodies were experiencing their inundated phase, a 200 µm mesh-sized hand net was used to sample along shorelines, through submerged vegetation and in open waters, paying attention to collect crustaceans in all the microhabitats present in each sampled site. Collected crustacean samples were fixed *in situ* using 90 % ethanol. Due to the different phenology of the species and to the seasonal succession of the crustacean assemblages of temporary waters, the presence of several taxa might be overlooked in sampling surveys with no or few temporal replicates; in such cases, the ex-situ re-hydration of dry sediment, known as "Sars' method" (van Damme & Dumont, 2010), is a useful complement to the study of the actual crustacean diversity occurring in a given area. Accordingly, sediment samples were collected from most of the sampled water bodies and cultured in laboratory. About 300 gr of sediment were collected from each water body, integrating sediment samples from the deepest parts and the shores. When collected wet, the sediment was first dried at room temperature in open plastic bags. Sediment subsamples from each site were then rehydrated in laboratory using 4-litres plastic aquaria where a thin layer of sediment (about 5 mm) was introduced with 3 litres of distilled water. Cultures were run at the University of Palermo during winter months and kept at temperature between 12 and 16 °C with a 12-hours photoperiod and a feeble aeration. Cultures were maintained for about two months and monitored each second day. The hatched crustaceans were collected monthly by filtering the water over a 125 µm plankton net. Some specimens belonging to poorly-represented species in the cultures were individually collected by pipetting. The procedure described above was repeated four times and designated as "Hydration Experiments" 1-4 (Table S1, available at http://www.limnetica.net/en/limnetica).

Crustacean samples collected in the wild or coming from laboratory cultures were sorted in laboratory under a dissecting stereomicroscope. Branchiopods were identified according to Alonso (1996), Kotov & Alonso (2010) and Sinev *et al.* (2012); copepods according to Dussart (1967, 1969), Kiefer (1971, 1978), Borutzky *et al.* (1991), Einsle (1993) and Ranga-Reddy (1994); ostracods according to Meisch (2000) and Mazzini *et al.* (2014).

Undissected crustacean specimens were stored in 95 % ethanol at -20 °C, dissected soft parts were stored in glycerol in sealed microscope slides, and ostracod valves were dried and stored in micropaleontological slides.

Voucher specimens of *Arctodiaptomus alpinus* (Imhof, 1885) from GR088 (MZUF634) and GR100 (MZUF635), and *Chirocephalus diaphanus* from GR094 (MZUF637) and GR099 (MZUF636), were deposited in the Museo di Storia Naturale, Sezione di Zoologia ''La Specola'', Università di Firenze, Italy (MZUF); other specimens are currently stored in FM's collection at the Dipartimento di Scienze e Tecnologie Biologiche, Chimiche e Farmaceutiche of the University of Palermo, Italy, and are available for loan on request.

Because of the unsettled systematics of the large branchiopod genus *Chirocephalus* and, in particular, of the species attributed so far to the *C. diaphanus*-group sensu Brtek (1995), two specimens of *Chirocephalus diaphanus* s.l. from sites GR084 and GR099 (Crete, Greece) were studied by amplifying and sequencing a fragment of the mitochondrial gene encoding for the cytochrome oxidase subunit I (COI), a molecular marker widely used in the molecular systematics and phylogeography of *Chirocephalus* (e.g.: Ketmaier *et al.*, 2012; Reniers *et al.*, 2013; Zarattini *et al.*, 2013; Cottarelli *et al.*, 2017). Comparative

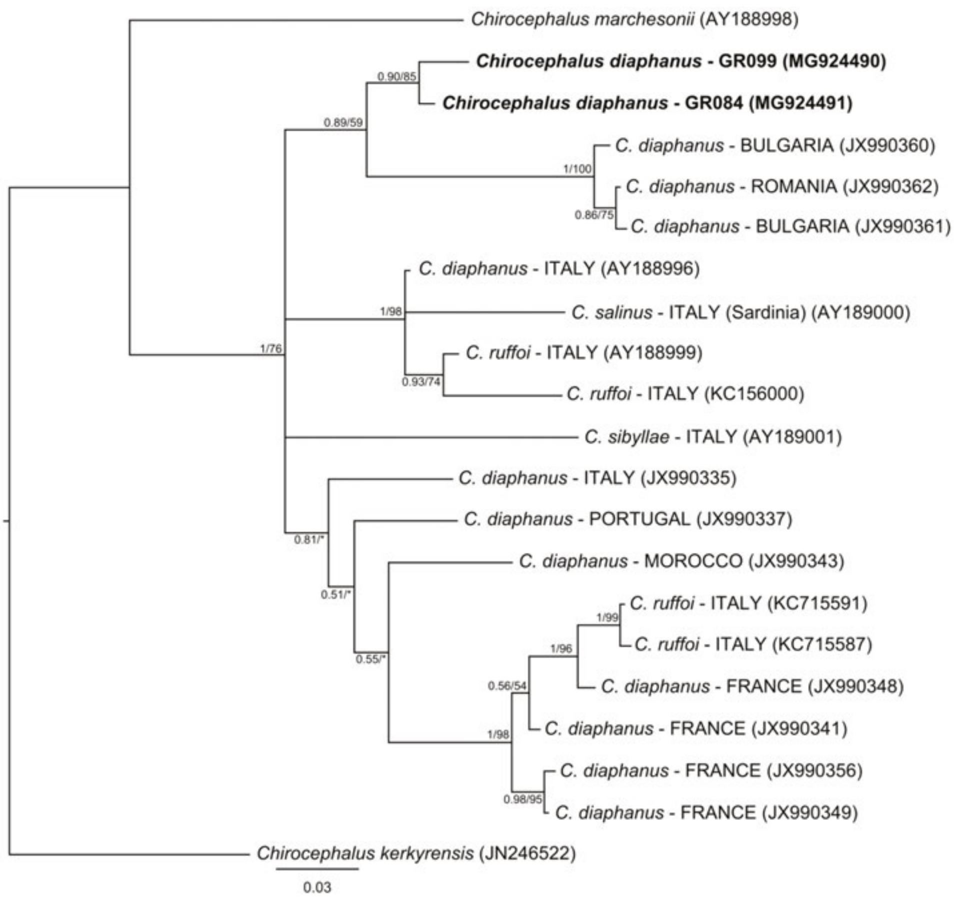

Figure 2. Bayesian consensus phylogram based on a 408-bp-long fragment of the mitochondrial gene encoding for the cytochrome oxidase subunit I (mtDNA COI). Node support is reported as nodal posterior probability/ML bootstrap; nodal posterior probability lower than 0.5 is represented as an unresolved polytomy; "*" indicates bootstrap support lower than 50. Accession numbers of sequences derived from GenBank are shown in brackets. Cretan samples are reported in bold. *Árbol filogenético de consenso Bayesiano basado en el fragmento largo 408-bp del gene mitocondrial codificado por la subunidad I citocromo oxidasa (mtDNA COI). El soporte de los nodos se muestra como la probabilidad posterior nodal /ML bootstrap; la probabilidad posterior nodal inferior a 0.5 se representa como una politomía no resuelta. "*" indica un soporte boostrap inferior a 50. Entre paréntesis los números de acceso de las secuencias derivadas del GenBank. En negrita las muestras de Creta.*

sequences belonging to other *C. diaphanus* s.l. populations and other *Chirocephalus* species were downloaded from GenBank and included in the analyses (Fig. 2).

Calanoid copepods were prepared according to Dussart and Defaye (2001), and line drawings of specimens of the genus *Arctodiaptomus* were made using a compound microscope equipped with a camera lucida. Drawings were based on specimens collected in GR084, an ancient temporary pond located on the Omalos plateau (Chania, Crete). Two specimens of *Arctodiaptomus* cf. *piliger* from sites GR088 and GR099 (Crete, Greece), one specimens of *A. alpinus* from Greek mainland (Central Greece), one specimen of *Arctodiaptomus* cf. *alpinus* from Mongolia (Tov province), one specimen of *Arctodiaptomus belgrati* Mann, 1940 from Romania (Ciuc basin), and one specimen of *Arctodiaptomus kerkyrensis* Pesta, 1935 from Italy (Basilicata) were studied by amplifying and sequencing a fragment of the mitochondrial gene encoding for the cytochrome b (Cyt-b). Cyt-b sequences proved to be informative about the molecular systematics of the genus

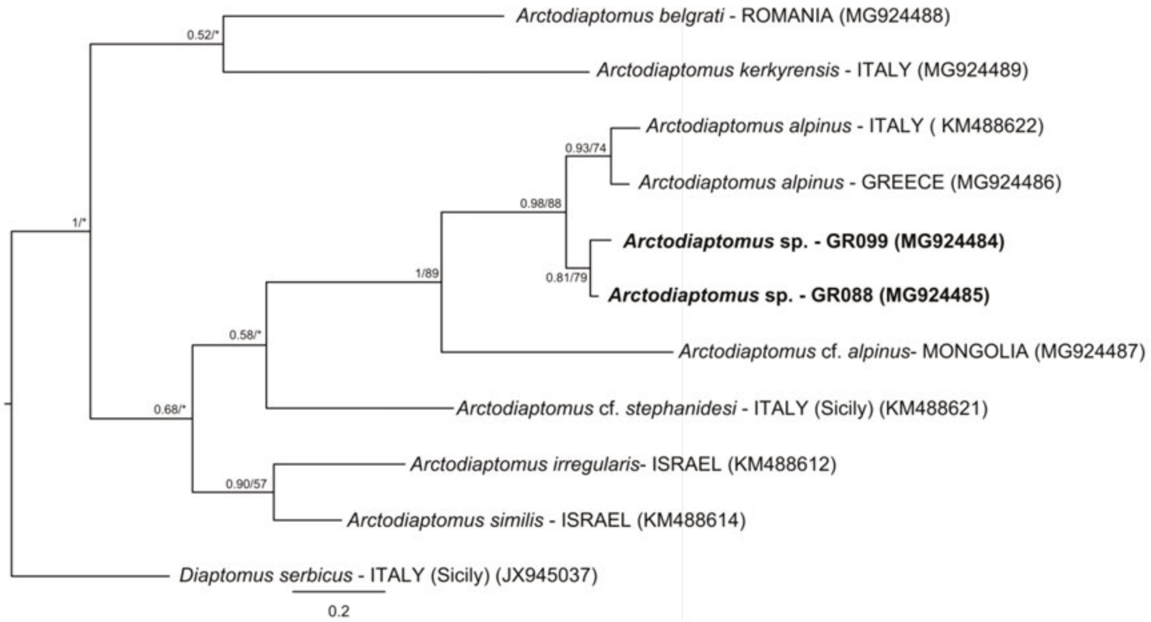

Figure 3. Bayesian consensus phylogram based on a 313-bp-long fragment of the mitochondrial gene encoding for the cytochrome b (mtDNA Cyt-b). Node support is reported as nodal posterior probability/ML bootstrap; nodal posterior probability lower than 0.5 is represented as an unresolved polytomy, "*" indicates bootstrap support lower than 50. Accession numbers of sequences derived from GenBank are shown in brackets. Cretan samples are reported in bold. *Árbol filogenético de consenso Bayesiano basado en el fragmento largo 313-bp del gene mitocondrial codificado por la subunidad I citocromo oxidasa (mtDNA Cyt-b). El soporte de los nodos se muestra como la probabilidad posterior nodal /ML bootstrap; la probabilidad posterior nodal inferior a 0.5 se representa como una politomía no resuelta. "*" indica un soporte boostrap inferior a 50. Entre paréntesis los números de acceso de las secuencias derivadas del GenBank. En negrita las muestras de Creta.*

Arctodiaptomus (Marrone *et al.*, 2014); accordingly, this molecular marker was chosen with the aim of comparing the allegedly endemic Cretan species *Arctodiaptomus piliger* Brehm, 1955 and different populations of the closely related *A. alpinus* (Imhof, 1885). Moreover, comparative sequences belonging to an *A. alpinus* population from the Italian Alps and other species of the genera *Arctodiaptomus* and *Diaptomus* were downloaded from GenBank and used in the analyses (Fig. 3).

DNA extraction and amplification were performed following the protocols and procedures described in Marrone *et al.* (2010, 2013) for copepods, and Cottarelli *et al.* (2017) for anostracans. Chromatograms were visualised and edited using Chromas Lite 2.01 (Technelysium Pty Ltd., South Brisbane, Australia), and aligned in ClustalX (Thompson *et al.*, 1997). MEGA7 (Kumar *et al.*, 2016) was used to translate the Cyt-b and COI sequences to amino acids in order to check for the possible presence of frameshifts or stop codons, which would indicate the presence of sequencing errors or pseudogenes. The sequences were deposited in GenBank under Accession Numbers MG924484-MG924489 (*Arctodiaptomus* spp.) and MG924490-MG924491 (*Chirocephalus diaphanus* s.l.).

Bayesian inference (BI) of phylogeny and maximum likelihood (ML) analyses were performed on the Cyt-b dataset as implemented by MrBayes 3.2 (Ronquist *et al.*, 2012) and PhyMl v.3 (Guindon & Gascuel, 2003), respectively. The choice of the best evolutionary model was made using MrModeltest 2.2 (Nylander 2004) on the basis of Akaike Information Criterion (AIC) for both analyses and datasets; accordingly, a Hasegawa-Kishino-Yano nucleotide substitution model with gamma distributed rate variation among sites (HKY+G; nst=2) was used for the "*Chirocephalus* dataset", and a General Time Reversible model with gamma distributed rate variation among sites and a proportion of invariant sites (GTR+I+G; nst=6) was used for the "*Arctodiaptomus* dataset". Node supports were evaluated by their posterior probabilities in the BI and by 1000 bootstrap replicates in the ML analyses. The BI analyses were performed with two independent runs of 1 x 10^6 generations and four Markov chains using default heating values. Trees and parameter values were sampled every 100 generations resulting in 10 x 10^3 saved trees per analysis. An initial fraction of 2000 trees (20 %) was conservatively discarded as *burn-in*. For all analyses and both datasets, standard deviation of split frequencies reached values lower than 0.018288, and values of the potential scale reduction factor (PSRF) were comprised between 0.999 and 1.002 for all the partitions with frequency ≥ 0.10 in at least one run, indicating convergence of the runs.

RESULTS

Four permanent and 17 temporary water bodies spread throughout Crete, from sea level up to 1330 m a.s.l., were sampled (Fig. 1 and Table 1; Table S2 and Fig. S1 available at http://www.limnetica.net/en/limnetica). Zooplankton samples were available from 8 sites only; sediment samples were collected in further 10 sites, since these were dry when sampling was performed. The crustacean fauna occurring in 3 additional sites was studied based both on direct crustacean samplings and sediment culturing (Table 1; Table S1 available at http://www.limnetica.net/en/limnetica).

Overall, 46 non-malacostracan crustacean taxa (17 Branchiopoda, 10 Copepoda, and 19 Ostracoda) were collected in the present study, and 27 of these are new for the Cretan fauna. These results therefore bring to 78 the number of non-malacostracans species up to now known for the island (Table 2). Most of the collected taxa were identified at species level, although this proved not to be possible for the cladocerans *Ilyocryptus* sp. from GR083 and GR094 and *Leydigia* sp. from GR084, and the ostracods *Cypridopsis* sp. (from GR152), *Candona* sp. (from GR094), *Ilyocypris* sp. (from GR089, GR094, GR099), and *Eucypris* sp. occurring in GR092 and GR151.

A single anostracan species morphologically ascribed to *Chirocephalus diaphanus* Prévost, 1803 was found in five of the studied sites (Table 2; Fig. S2 available at http://www.limnetica.net/en/limnetica). Upon aligning our Cretan *Chirocephalus* sequences and those downloaded from GenBank, and having trimmed the tails of the sequences which were not present in all the individuals, we obtained a properly aligned 408 bp-long fragment of the mtDNA COI. BI and ML trees, rooted on *Chirocephalus kerkyrensis* Pesta, 1936 (a species belonging to the *bairdi*-species group of the genus *Chirocephalus*, see discussion in Cottarelli *et al.*, 2017), showed a consistent topology at the major nodes (Fig. 2). The sequences of the Cretan *Chirocephalus* specimens form the sister clade of *C. diaphanus* s.l. sequences from Bulgaria and Romania, i.e. were included in the "eastern clade" of *C. diaphanus* s.l., which should be ascribed to the binomen *Chirocephalus romanicus* Stoicescu, 1992 according to Reniers *et al.* (2013).

The widespread *Ceriodaphnia* sp., observed in 6 out of the 21 studied sites, corresponds to the taxon ascribed to *Ceriodaphnia quadrangula* Müller, 1785 by Alonso (1996). However, this is, in fact, a different widespread west-Palearctic species pending a formal description (M. Alonso, unpublished data).

Three species of calanoid copepods were collected in the frame of this survey: *Calanipeda aquaedulcis* Kritschagin, 1873, *Neolovenula alluaudi* (Guerne & Richard, 1890), and a diaptomid belonging to the genus *Arctodiaptomus*. The morphology of both males and females of the latter species are presented in figures 4 and 5, respectively.

Five cyclopoid and two harpacticoid species were collected during this survey (Table 2). Among these, the cyclopoids *Acanthocyclops einslei* Mirabdullayev & Defaye, 2004, the widespread *Metacyclops minutus* (Claus, 1863), and the brackish water harpacticoid *Cletocamptus retrogressus* Schmankevitsch, 1875 are new records from Crete.

Table 2. Checklist of the non-stygobitic taxa cited for Crete island; codes of the sites of occurrences according to table 1. References: 1: Kiefer (1928); 2: Chappuis (1929); 3: Klie (1941); 4: Brehm (1955); 5: Lindberg (1956); 6: Chappuis (1956); 7: Pesce & Maggi (1981); 8: Petkowski *et al.* (2000); 9: Karaouzas *et al.* (2000); 10: Present work.; [1]: “*C. quadrangula*” *sensu* Alonso (1996) and probably *sensu* Brehm (1955); [2]: described as *A. piliger* by Brehm (1955); [3]: uncertain identification; cited as *Cyclops strenuus* (Chappuis, 1929) and *Cyclops rubens rubens* (Lindberg, 1956); [4]: reported as *Cyclops bicuspidatus odessanus* (Chappuis, 1929) and *Acanthocyclops bicuspidatus* f. *odessana* (Lindberg, 1956). *Relación de los taxones no estigobíticos citados en la isla de Creta; códigos de las localidades donde aparecen de acuerdo con la tabla 1. Referencias: 1: Kiefer (1928); 2: Chappuis (1929); 3: Klie (1941); 4: Brehm (1955); 5: Lindberg (1956); 6: Chappuis (1956); 7: Pesce & Maggi (1981); 8: Petkowski* et al. *(2000); 9: Karaouzas* et al. *(2000); 10: Present work.; [1]:* “C. quadrangula*”* sensu *Alonso (1996) y probablemente* sensu *Brehm (1955); [2]: descrito como* A. piliger *por Brehm (1955); [3]: identificación dudosa; citado como* Cyclops strenuus *(Chappuis, 1929) y* Cyclops rubens rubens *(Lindberg, 1956); [4]: reportado como* Cyclops bicuspidatus odessanus *(Chappuis, 1929) y* Acanthocyclops bicuspidatus *f.* odessana *(Lindberg, 1956).*

Taxa	References	Sites of occurrence (Present work)
BRANCHIOPODA		
Anostraca		
Chirocephalidae		
Chirocephalus diaphanus s.l. Prévost, 1803	9, 10	GR084, GR088, GR094, GR099, GR175
Anomopoda		
Daphniidae		
Daphnia (Ctenodaphnia) atkinsoni Baird, 1859	10	GR151
Daphnia (Ctenodaphnia) chevreuxi Richard, 1896	10	GR088, GR094, GR099
Simocephalus vetulus (Müller, 1776)	4, 10	GR084, GR087, GR100
Ceriodaphnia sp.[1]	4, 10	GR084, GR087, GR088, GR094, GR099, GR152
Ceriodaphnia reticulata (Jurine, 1820)	4, 10	GR085
Moinidae		
Moina brachiata (Jurine, 1820)	10	GR099
Moina micrura Kurz, 1875	10	GR084, GR088
Ilyocryptidae		
Ilyocryptus sp.	10	GR083, GR094
Macrothricidae		
Macrothrix hirsuticornis Norman & Brady, 1867	10	GR084, GR088, GR094, GR099, GR100, GR151
Chydoridae		
Chydorus sphaericus (Müller, 1776)	4, 5, 10	GR084, GR100
Ovalona anastasia (Sinev, Alonso, Miracle & Sahuquillo, 2012)	10	GR084
Ovalona nuragica (Margaritora, 1971)	10	GR084
Alona affinis (Leydig, 1860)	4, 5	---
Alona salina Alonso, 1996	10	GR091, GR096
Alonella excisa (Fischer, 1854)	4, 5	---
Alonella exigua (Lilljeborg, 1853)	5	---
Alonella nana (Baird, 1843)	5	---
Coronatella rectangula (G.O. Sars, 1862)	10	GR085, GR087, GR097, GR099, GR100
Leydigia iberica Kotov & Alonso, 2010	10	GR088
Leydigia sp.	10	GR084
COPEPODA		
Calanoida		
Pseudodiaptomidae		
Calanipeda aquaedulcis Kritschagin, 1873	4, 10	GR082
Diaptomidae		
Arctodiaptomus (*Rhabdodiaptomus*) *alpinus* (Imhof, 1855)[2]	4, 10	GR084, GR088, GR099, GR100, GR152
Neolovenula alluaudi (Guerne & Richard, 1890)	7, 10	GR084, GR085
Cyclopoida		
Cyclopidae		
Macrocyclops albidus (Jurine, 1820)	5	---
Eucyclops serrulatus (Fischer, 1851)	2, 5, 7	---
Ectocyclops phaleratus (Koch, 1838)	5	---
Tropocyclops prasinus (Fischer, 1860)	2, 5, 10	GR085, GR087
Paracyclops fimbriatus (Fischer, 1853)	5, 7	---

Cont.

Table 2. (cont.)

Cyclops sp.[3]	2, 5	---
Megacyclops viridis (Jurine, 1820)	5, 7, 10	GR085
Acanthocyclops einslei Mirabdullayev & Defaye, 2004	10	GR083
Diacyclops bisetosus (Rehberg, 1880)	7	---
Diacyclops lubbocki (Brady, 1869)[4]	2, 5, 7, 10	GR086, GR091
Diacyclops crassicaudis (G.O. Sars, 1863)	1, 2, 7	---
Thermocyclops oblongatus (G.O. Sars, 1927)	5, 7	---
Metacyclops minutus (Claus, 1863)	10	GR098, GR151
Harpacticoida		
Canthocamptidae		
Mesochra aestuarii Gurney, 1921	6	---
Canthocamptus staphylinus (Jurine, 1820)	2, 6, 10	GR084
Attheyella (Attheyella) crassa (Sars, 1863)	2	---
Bryocamptus (Bryocamptus) minutus (Claus, 1863)	2, 6	---
Bryocamptus (Rheocamptus) pygmaeus (Sars, 1863)	2, 6	---
Bryocamptus (Rheocamptus) zschokkei (Schmeil, 1893)	2	---
Elaphoidella denticulata Chappuis, 1929	2	---
Maraenobiotus vejdovskyi Mrazek, 1893	6	---
Maraenobotus brucei carpathicus Chappuis, 1928	6	---
Cletocamptus retrogressus Schmankevitsch, 1875	10	GR086, GR091, GR097
Ameiridae		
Nitokra lacustris (Shmankevich, 1875)	6	---
Laophontidae		
Onychocamptus mohammed (Blanchard & Richard, 1891)	6	---
OSTRACODA		
Podocopida		
Candonidae		
Candona lindneri Petkovski, 1969	10	GR088
Candona neglecta Sars, 1887	3	---
Candona sp.	10	GR094
Pseudocandona pratensis (Hartwig, 1901)	3	---
Candonopsis kingsleii (Brady & Robertson, 1870)	3	---
Ilyocypridae		
Ilyocypris sp.	10	GR088, GR094, GR099
Ilyocypris bradyi Sars, 1890	3	---
Ilyocypris divisa Klie, 1926	3	---
Ilyocypris gibba (Ramdohr, 1808)	3, 10	GR091
Ilyocypris getica Masi, 1906	10	GR100
Cyprididae		
Cypris bispinosa Lucas, 1849	10	GR083, GR087
Eucypris kerkyrensis Stephanides 1937	8	---
Eucypris virens (Jurine, 1820)	3, 10	GR091, GR099, GR100
Eucypris sp.	10	GR092, GR151
Tonnacypris lutaria (Koch, 1838)	8, 10	GR098, GR100, GR151
Herpetocypris chevreuxi (Sars, 1896)	3, 8, 10	GR084
Herpetocypris reptans Cole, 1965	3	---
Stenocypria fischeri (Lilljeborg, 1883)	10	GR100
Heterocypris barbara (Gauthier & Brehm, 1928)	10	GR086, GR094, GR097, GR099, GR100, GR151
Heterocypris incongruens (Ramdohr, 1808)	3, 8, 10	GR086, GR091, GR096, GR098, GR100, GR151
Heterocypris salina (Brady, 1868)	3, 8	---
Cypria ophtalmica (Jurine, 1820)	3	---
Cypridopsis vidua (O.F. Müller, 1776)	10	GR083
Cypridopsis hartwigi G.W. Müller, 1900	10	GR100
Cypridopsis elongata (Kaufmann, 1900)	10	GR100
Plesiocypridopsis newtoni (Brady & Robertson, 1870)	3	---
Sarscypridopsis aculeata (Costa, 1847)	10	GR085, GR091, GR097, GR152
Potamocypris arcuata (Sars, 1903)	10	GR088, GR094, GR098, GR099, GR100
Lymnocytheridae		
Paralimnocythere psammophila (Flössner, 1965)	10	GR100

Morphological description of the *Arctodiaptomus* specimens from Crete

Male: Body length 1.410 mm, shorter than in female (Fig. 4a). Urosome asymmetrical (Fig. 4b) with a strong spine on the right side of the first genital somite and scattered small setae (in variable number) on the other somites. Geniculate right antennula (Fig. 4e) with spines at the segment 8 and 10-13; length proportions as follows: 13>11>10>8>12. Antepenultimate segment of right antennula with a spinous process shorter than the penultimate segment and with a rounded-hyaline terminal end (Fig. 4f), variable in different specimens (Fig. 4g). Rostral spines as in Fig. 4h. Posterior surface of the second endopodite segment of leg 2 with a weakly sharpened "Schmeil's organ" (Fig. 4d). Right fifth leg (P5, Fig. 4c) with chitinous processes on the inner side of basis and on the second exopodite segment; distal claw with a proximal 'curve' as in *A. steindachneri* (Richard, 1897); lateral spine as long as one half of distal claw.

Female: Size 1.615 mm. Habitus as in Fig. 5a. Urosome slightly asymmetrical (Figs. 5b, 5c) with spines on both sides of the genital somite in variable position, but with the left spine always pointed down with respect to the right one. Antennula (Fig. 5l) with a long seta on the first segment ("dentifer") reaching the 8th segment. Two setae on segments 11 and 13, one seta on segments 15 and 17. P5 (Fig. 5d) with robust coxal spines and small sensilla variable both in number and position (Figs. 5e, 5g). Endopodite of fifth pair of legs indistinctly 2-segmented with setulae on the inner side (Figs. 5f, 5h, 5i); a setula can be present only on a single segment or in both the indistinct segments; variability was observed even in the same specimen in left and right legs (Figs. 5h, 5i).

By analysing the morphological features of the collected specimens, it was noticed that the morphological characters (and the drawings) originally used by Brehm (1955) to describe the species *Arctodiaptomus piliger* do not allow to distinguish it from the congeneric *A. alpinus* (Imhof, 1885). These results were confirmed by the molecular analyses based on a 313 bp-long fragment of the Cyt-b gene. In both the BI and ML trees (Fig. 3), which showed a concordant topology at the major nodes, Cretan *Arctodiaptomus* cf. *piliger* sequences clustered with *Arctodiaptomus alpinus* s.s. sequences from Italy and Greece. Conversely, *Arctodiaptomus* cf. *alpinus* reported for Mongolia by Marrone *et al.* (2015) forms the sister group of the studied European *A. alpinus* populations. The 26 % of uncorrected p-distance between the Mongolian sample and the European *A. alpinus* s.s. populations is even higher than that observed between the sister species *A. similis* (Baird, 1859) and *A. irregularis* Dimentman & Por, 1985 (Marrone *et al.*, 2014), thus suggesting that the Mongolian taxon might actually represent a cryptic species related to *A. alpinus* s.s.

DISCUSSION

To date, the microcrustaceans and large branchiopods of the inland waters of Crete were poorly known. While several papers dealing with the stygobitic copepods of Crete were published in the XX century (e.g. Chappuis, 1929, 1956; Lindberg, 1956; Pesce & Maggi, 1981, 1983 and references therein), the Cretan surface non-malacostracan crustaceans, to our knowledge, were the object of a few studies only (Chappuis, 1929; Klie, 1941; Brehm, 1955; Lindberg, 1956; Karaouzas *et al.*, 2015). Although the present work admittedly does not provide an exhaustive list of Cretan inland water non-malacostracan crustaceans, it paves the way to further studies, and allows to carry out a first characterization of Cretan fauna. The vast majority of observed taxa are first records for Crete and/or for the eastern Mediterranean basin, further stressing the need of better exploring these areas.

A single anostracan species was observed; this result, although unexpected when the anostracan fauna of other large Mediterranean islands is considered (Brtek & Thiéry, 1995), is in line with the paucity of large branchiopod records to date available for Greece (Abatzopoulos *et al.*, 1999). Its morphology is compatible with those described for the subspecific taxa *C. diaphanus carinatus* Daday, 1910 (*locus typicus*: valley of river Vardar\Axios, Greece), *C. diaphanus pentheri* Pesta, 1921 (*locus typicus*: Kukës, Alba-

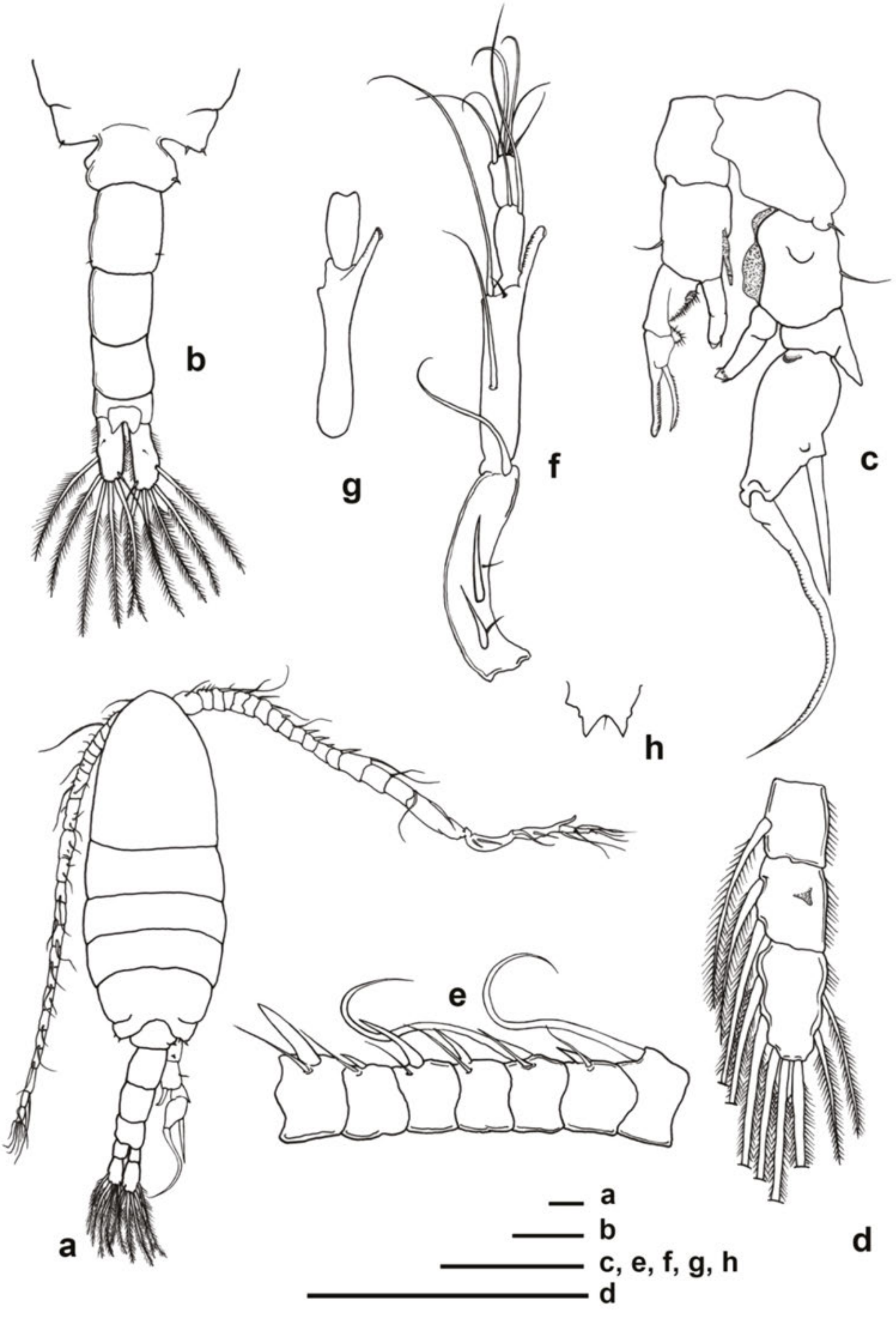

Figure 4. *Arctodiaptomus alpinus* from Crete (GR084); Male. a: habitus; b: urosome; c: fifth pair of legs; d: second leg; e: right antennula (segments 7-13); f: antepenultimate segment of the right antennula; g: as in f (different specimen). Scale bar: 0.1 mm. Drawings by GA. Arctodiaptomus alpinus *de Creta (GR084); Macho. a: habitus; b: urosoma; c: quinto par de patas; d: segunda pata; e: anténula derecha (segmentos 7-13); f: antepenúltimo segmento de la anténula derecha; g: como en f (ejemplar diferente). Barra de escala: 0.1 mm. Ilustraciones de GA.*

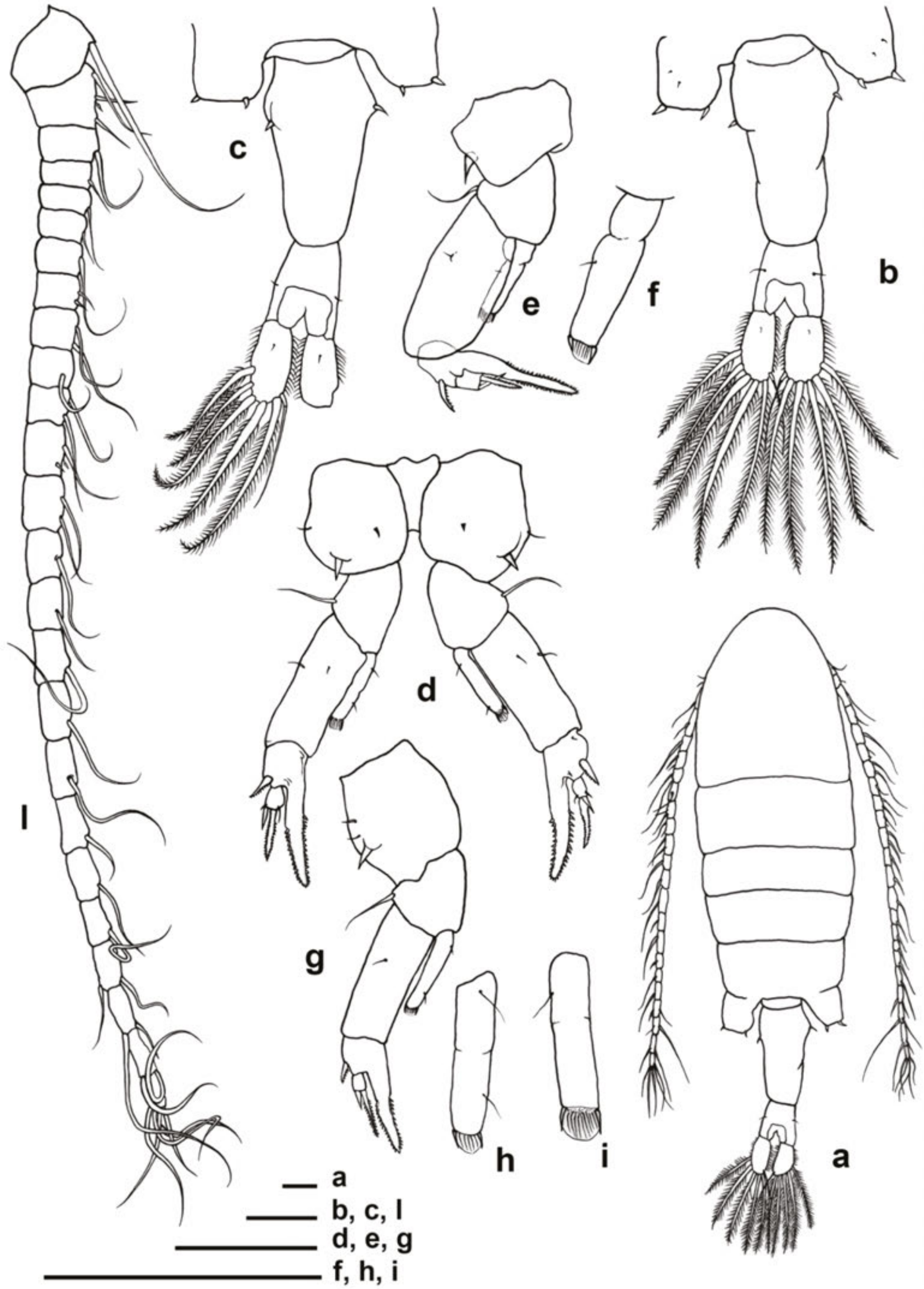

Figure 5. *Arctodiaptomus alpinus* from Crete (GR084); Female. a: habitus; b, c: urosome; e, d, g: fifth pair of legs; f, h, i: endopodite of fifth leg; l: A1. (Specimen 1: a, b, d, l; Specimen 2: c, g, h, I; Specimen 3: e, f). Scale bar: 0.1 mm. Drawings by GA. Arctodiaptomus alpinus *de Creta (GR084); Hembra. a: habitus; b, c: urosoma; e, d, g: quinto par de patas; f, h, i: endopodito de la quinta pata; l: A1. (Ejemplare 1: a, b, d, l; Ejemplare 2: c, g, h, I; Ejemplare 3: e, f). Barra de escala: 0.1 mm. Ilustraciones de GA.*

nia) and *C. diaphanus romanicus* Stoicescu, 1962 (*locus typicus*: Oltenița, Romania), although, as stressed by several authors (e.g. Belk & Brtek, 1995; Marincek & Petrov, 1995; Mura *et al.*, 2002; Reniers *et al.*, 2013) a proper distinction of *C. diaphanus* subspecies is not always straightforward, and a revision of this species-group is needed. The molecular analyses of the Cretan *Chirocephalus* from GR084 and GR099 clustered the two analysed specimens within the "eastern clade" of *C. diaphanus*, which included samples from Romania and Bulgaria. According to Reniers *et al.* (2013) this clade should be worth of species status under the binomen *Chirocephalus romanicus*. However, in the light of the wide morphological plasticity of *C. diaphanus* s.l. and of the morphological similarity among its eastern subspecies, "*C. romanicus*" might in fact prove to be just a junior synonym of *C. pentheri* or *C. carinatus*. Accordingly, pending a revision of the systematics and taxonomy of the *diaphanus*-group of the genus *Chirocephalus*, which includes samples from the type localities of all the described taxa, we prefer to report the Cretan *Chirocephalus* as *C. diaphanus* s.l., refraining from assigning it to any other taxon of specific or subspecific rank. In Greece, *C. diaphanus* s.l. was already reported for Zante (Stephanides, 1948), Boeotia (Pesta, 1921) and Macedonia (Daday, 1910). Moreover, Karaouzas *et al.* (2015) report the presence of *Chirocephalus* sp. from Omalos temporary pond (Crete), i.e the site coded as GR084 in the present study.

In the last decades, careful morphological studies confirmed that, as anticipated by Frey (1982), the paradigm of cosmopolitanism of freshwater taxa was inapplicable to anomopods, and that their actual diversity was largely underestimated (Forró *et al.*, 2008). Accordingly, several new species were recently described, even in well-studied regions, especially among the radopods. These taxa, originally considered endemic to their *terra typica*, are now being observed even in relatively-distant regions, thus better defining their distribution. This is the case for some alleged west-Mediterranean endemic taxa as *Ovalona anastasia* (Sinev, Alonso, Miracle & Sahuquillo, 2012), *Ovalona nuragica* (Margaritora, 1971), *Alona salina* Alonso, 1996 and *Leydigia iberica* Kotov & Alonso, 2010, whose presence on an island of the eastern Mediterranean Sea was rather unexpected and further stresses the need for a careful re-examination of the anomopod fauna of the eastern Mediterranean countries.

The observed Cretan copepod fauna is rather poor when compared to other large Mediterranean islands (e.g. Sicily, Corsica, or Sardinia). These are usually inhabited by 6-11 diaptomid species, and even the relatively-small Balearic Islands and Corfu respectively host five and six diaptomid species (Marrone *et al.*, 2009). These numbers are significantly higher than the two diaptomid species observed on Crete. This paucity is likely to be ascribed to the inadequacy of sampling and/or of the used culturing techniques for the hatching or raising of calanoid copepods. Actually, Crete has a climate, geographic location and physiography which should allow the presence of a much richer diaptomid fauna (Marrone *et al.*, 2017). The same applies to cyclopoid and harpacticoid copepods, whose observed species richness is almost completely due to the few samples collected during the wet phase of the water bodies, while only three species, i.e. *Diacyclops lubbocki* (Brady, 1869), *Metacyclops minutus* (Claus, 1863) and *Cletocamptus retrogressus* Schmankevitsch, 1875 hatched in the cultures (see Table S1, available at http://www.limnetica.net/en/limnetica). It is then obvious that different culturing methods are to be developed and implemented to efficiently use the Sars' method for censing the copepods occurring in a given area. Until culturing methodologies are optimised, the study of copepods should be primarily based on collections in the field. It is likely that an increased sampling effort will lead to the finding of several other copepod taxa, including representatives of the diaptomid genera *Mixodiaptomus* and *Hemidiaptomus* which are among the most typical copepod inhabitants of Mediterranean temporary ponds (Miracle, 1982; Sahuquillo & Miracle, 2013).

The three calanoid copepods recorded in the frame of this survey were already known to occur on the island (Brehm, 1955; Pesce & Maggi, 1981; Alfonso & Belmonte, 2013). Interestingly, we found abundant populations of the alleged Cretan endemic diaptomid *Arctodiaptomus* *piliger* in five temporary ponds spread throughout the island. The species was only sketchy described by Brehm (1955) and never collected again since its original description so that providing more details on its morphology and ecology was considered pivotal according to Kiefer (1978), Ranga-Reddy (1994) and Błędzki & Rybak (2016). *Arctodiaptomus piliger* was ascribed to the *bacillifer*-group sensu Kiefer (1971) based on the occurrence of two setae on the 13th antennulary segment. According to the original description, the only morphological character which would be discriminative of *A. piliger* and which would have differentiated this taxon from the closely-related *A. alpinus* was the presence of "long hairs" on the endopodites of female fifth pair of legs. This character, however, has no taxonomic value since it is routinely reported for most *Arctodiaptomus* species, including *Arctodiaptomus alpinus* (cf. Dussart, 1967; Petkovski, 1983; Stella, 1984; Kiefer, 1971, 1978; and personal observations). Moreover, the molecular identification of Cretan *Arctodiaptomus* specimens from two sites located more than 210 km apart on the island, consistently showed their conspecificity with *Arctodiaptomus alpinus* populations from the Italian Alps and from Central Greece. Morphological re-examination and molecular analyses of alleged *Arctodiaptomus piliger* are thus concordant in supporting the synonymization of this species with the widespread *Arctodiaptomus alpinus*, a species also occurring on mainland Greece (present data) and spread throughout the Palearctic (Dussart & Defaye, 2002; Flössner *et al.*, 2005; but see also: Marrone *et al.*, 2015), which is here formally indicated as the senior synonym of *A. piliger*. The possible occurrence of *Arctodiaptomus alpinus* on Crete suggested by Kiefer (1971, pag. 148) and reported (but not discussed) by Ranga-Reddy (1994), Jersabek *et al.* (2011, Fig.7) and Błędzki & Rybak (2016, figure at pag. 491) is thus here fully confirmed. Our findings provide some unprecedented information on the ecology of the species, which was usually considered a "cold stenothermal species, occurring preponderantly in the lakes of high places in the Alps (altitude 2000-2700 m)" (Ranga-Reddy, 1994) and a "real high-altitude species with a strong preference for ultraoligotrophic alpine lakes above the timberline" (Błędzki & Rybak, 2016). Conversely, in Crete the species was collected in eutrophic temporary ponds and pools frequented by livestock, and located in the range 539-1330 m a.s.l. In some cases, it co-occurred with *Neolovenula alluaudi*, a Paradiaptominae copepod considered typical of steppic regions (Jaume, 1989-1990; Alfonso & Belmonte, 2013).

All the cyclopoid and harpacticoid species collected so far from surface waters in Crete are widespread in the Mediterranean bioregion as well as across the Palearctic, confirming their high dispersal and colonization ability. *Metacyclops minutus* was found so far only in temporary ponds and pools, showing a preference for small and ephemeral water bodies (Dussart, 1969; Einsle, 1993); it was the species most commonly obtained by the "Hydration Experiments".

To our knowledge, Klie (1941) and Petkowski *et al.* (2000) were the only authors who provided data on the ostracod fauna of Crete, altogether listing 15 species; among these, 10 were not found in the present study. Accordingly, considering both the data of Klie (1941) and the present data, the ostracod fauna of Crete at present includes 29 taxa. Among ostracods, the findings of *Ilyocypris* sp., a taxon already recorded from Greece (Mazzini *et al.*, 2014), peninsular Italy (Alfonso *et al.*, 2016) and Tunisia (unpublished data), and *Eucypris* sp. are noteworthy. These taxa are currently under study and might be species new to Science pending a formal description. Moreover, the specific allocation of both *Candona* sp. and *Cypridopsis* sp. remained uncertain, due to either absence of males or presence of exclusively juvenile specimens.

Based on the currently available data, the non-malacostracan crustacean fauna of the inland waters of Crete is a typically Mediterranean one, including both steppic and more temperate taxa. However, the well-developed orography of the island also allows the presence of species, as the calanoid copepod *Arctodiaptomus alpinus*, which are typical of colder climates. These organisms might represent in Crete relic taxa which colonised the island during Pleistocene glaciations and found at higher altitudes microthermal refuges where to survive the Holocene climate warming. Interestingly, in accordance to what observed in other large Mediterranean islands, no endemic taxa were found among the species inhabiting surface water bodies, while the microcrustacean stygofauna of Crete and other Mediterranean islands is well-diversified and characterised by the presence of strict endemics like the cyclopoid copepods of the genus *Speocyclops* (Lindberg, 1955, 1956; Pesce & Maggi, 1983) or the harpacticoid copepods of the genus *Elaphoidella* (Chappuis, 1929, 1956). This pattern is likely to be ascribed to the high potential for passive dispersal shown by organisms producing resting stages, and to their consequent ability of both rapidly colonising newly suitable habitats and likely maintaining an effective gene-flow among populations (see Kappas *et al.*, 2017), although some contrasting evidences on this topic are also available (Incagnone *et al.*, 2015).

The occurrence in Crete of several anomopod species currently considered endemic of the western Mediterranean area highlights the importance of the so-called Wallacean shortfall (Whittaker *et al.*, 2005) for certain "less charismatic" crustacean groups as the non-malacostracan crustaceans, and its bearing on our current understanding of their diversity patterns and biogeography.

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Zooplankton community structure from tropical temporary ponds during a flood period

Karime de Araujo Paina[1,2,*] and Maria da Graça Gama Melão[2]

[1] Postgraduate Program in Ecology and Natural Resources (PPG-ERN), Federal University of São Carlos (UFSCar), Rodovia Washington Luís, Km 235, Zip Code 13565-905, São Carlos, SP, Brazil.
[2] Department of Hydrobiology, UFSCar, Rodovia Washington Luís, Km 235, Zip Code 13565-905, São Carlos, SP, Brazil.

* Corresponding author: karime_paina@hotmail.com

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ABSTRACT

Zooplankton community structure from tropical temporary ponds during a flood period

Many ways of measuring ecosystem biodiversity have been used. Besides taxonomic diversity, the functional diversity approach is considered highly predictive of the structure of biological communities and ecosystem processes. Temporary aquatic ecosystems have a great evolutionary and ecological importance, as they are very endemic places with rare species, often endangered by local extinction. The objective of this study was to describe the structure of the zooplankton community of eight Brazilian temporary ponds during the flood period. The organism collections and measures of environmental variables were carried out from 01/31 to 02/02/2017. As they are small and ephemeral environments, with very peculiar characteristics, both the taxonomic diversity (27 taxa) and the functional one were relatively low, suggesting convergence of the functional characteristics of the species. The zooplankton community was divided into five functional groups and the most significant functional attributes in the grouping of the species were habitat, trophic group and food habit. The functional groups were similar to the taxonomic groupings, except for raptorial carnivorous (Copepoda Cyclopoida and one Rotifera) that comprised the same functional group. In terms of biomass, two species of the Anostraca of the genus *Dendrocephalus* far exceeded the other groups. It was shown that the presence of these large Branchiopoda has an impact on the composition and numerical density of the zooplankton community, particularly the Cladocera, possibly due to niche overlap.

Key words: *Dendrocephalus brasiliensis*, *Dendrocephalus thieryi*, Neotropics, biomass, taxonomic diversity, functional diversity, Brazilian savanna, ephemeral ecosystems

RESUMO

Estrutura da comunidade zooplanctônica em lagoas temporárias tropicais durante um período de cheia

Diversas maneiras de mensurar a biodiversidade dos ecossistemas têm sido utilizadas. Além da diversidade taxonômica, a abordagem da diversidade funcional é considerada altamente preditiva da estrutura das comunidades biológicas e dos processos ecossistêmicos. Ecossistemas aquáticos temporários possuem uma grande importância evolutiva e ecológica, sendo locais de muito endemismo que abrigam espécies raras, muitas vezes ameaçadas de extinção local. O objetivo deste estudo foi descrever a estrutura da comunidade zooplanctônica de oito lagoas temporárias brasileiras durante um período de cheia. As coletas dos organismos e medidas de variáveis ambientais foram realizadas de 31/01 a 02/02/2017. Por se tratar de ambientes pequenos e efêmeros, com características muito peculiares, tanto a diversidade taxonômica (27 táxons) quanto a funcional foram relativamente baixas, sugerindo convergência das características funcionais das espécies. A comunidade zooplanctônica foi dividida em cinco grupos funcionais, sendo que os atributos funcionais mais significativos no agrupamento das espécies foram o habitat, o grupo trófico e a hábito alimentar. Os grupos funcionais foram parecidos com os agrupamentos taxonômicos, exceto para os carnívoros raptoriais (Copepoda Cyclopoida e um Rotifera) que compuseram um mesmo grupo funcional. Em termos de biomassa, duas espécies do Anostraca do gênero Dendrocephalus *ultrapassaram em muito os outros grupos. Foi* *evidenciado que a presença desses grandes Branchiopoda impacta a composição e a densidade numérica da comunidade zooplanctônica, particularmente dos Cladocera, possivelmente pela sobreposição de nichos.*

Palavras chave: Dendrocephalus brasiliensis, Dendrocephalus thieryi, *Neotrópicos, biomassa, diversidade taxonômica, diversidade funcional, cerrado, ecossistemas efêmeros*

INTRODUCTION

Temporary aquatic ecosystems are of great evolutionary and ecological importance, having communities that are physiologically adapted and that have developed effective and diversified mechanisms for their colonization, permanence and reproduction. They are very endemic places that harbor rare species, which are often threatened with extinction. Although often neglected, these ecosystems are often "hot spots" of the biodiversity in a region or landscape (Céréghino *et al.*, 2008).

Temporary ponds are very common in arid and semi-arid regions (Brendonck *et al.*, 2008; Atashbar *et al.*, 2014) and are characterized by well defined and predictable periodic dry and flood cycles (Williams, 1996). During floods, characteristic aquatic communities develop (Pérez-Bilbao *et al.*, 2015). Their fauna and flora has physiological, morphological and behavioral adaptations in response to flood oscillations, making these environments strong points of speciation (Williams, 1996). To adapt to temporary conditions, organisms basically develop two strategies: to withstand the dry phase through dormant life stages or to migrate at the beginning of the dry season (Wiggins *et al.*, 1980). In the case of the invertebrate community, predominant in temporary ecosystems, many species guarantee the survival of offspring through diapause eggs. They are r-strategist species, with high reproduction capacity, rapid growth and short life cycles (Williams, 1997).

Despite the importance of temporary aquatic ecosystems and their vulnerability to biodiversity loss, there is little information about them in tropical regions. This lack of information, as well as inadequate management contribute to their deterioration and even disappearance. As they are small, shallow and temporary, they are often considered unproductive areas where disease-transmitting insects live. This, as well as the need for agricultural expansion, tend to cause a reduction of these habitats (Perez-Bilbao & Garrido, 2009; Pérez-Bilbao *et al.*, 2015). The establishment of invasive species, pollution caused by using fertilizers and pesticides, changes in hydrological functioning and climate change are some of the factors that threaten the integrity of this type of ecosystem, which can have an impact on the richness, abundance, dominance and biomass of their communities (Crossetti & Bicudo, 2005, Pérez-Bilbao *et al.*, 2015).

In Brazil, the semi-arid region of the northeast concentrates the largest number of this type of ecosystems due to the low rainfall and high rate of evaporation. However, environmental and anthropogenic changes, which cause an imbalance in the hydrological regime, contribute to desertification processes, which may lead to their extinction (Maltchik & Medeiros, 2006). Thus, understanding the functioning of these systems and the patterns of changes in their biological diversity is critical to their preservation as many of them are threatened by human activities or by climate change.

Biological diversity can be measured by taxonomic and functional approaches. The latter considers behavioral, physiological, morphological and life cycle characteristics, which are called functional traits of the species (Violle *et al.*, 2007; Webb *et al.*, 2010; Litchman *et al.*, 2013) and is considered highly predictive of the structure of biological communities and ecosystem processes (Mcgill *et al.*, 2006; Smith *et al.*, 2013).

Although zooplankton plays a major role in aquatic ecosystems as primary consumers, influencing the entire trophic chain, there are no studies about its functional traits in temporary ecosystems in Brazil. Passos (2012; 2017) studied the zooplankton community structure of 25 shallow temporary ponds from the same region of this study, but only under the taxonomic approach.

Thus, knowing the composition and variations

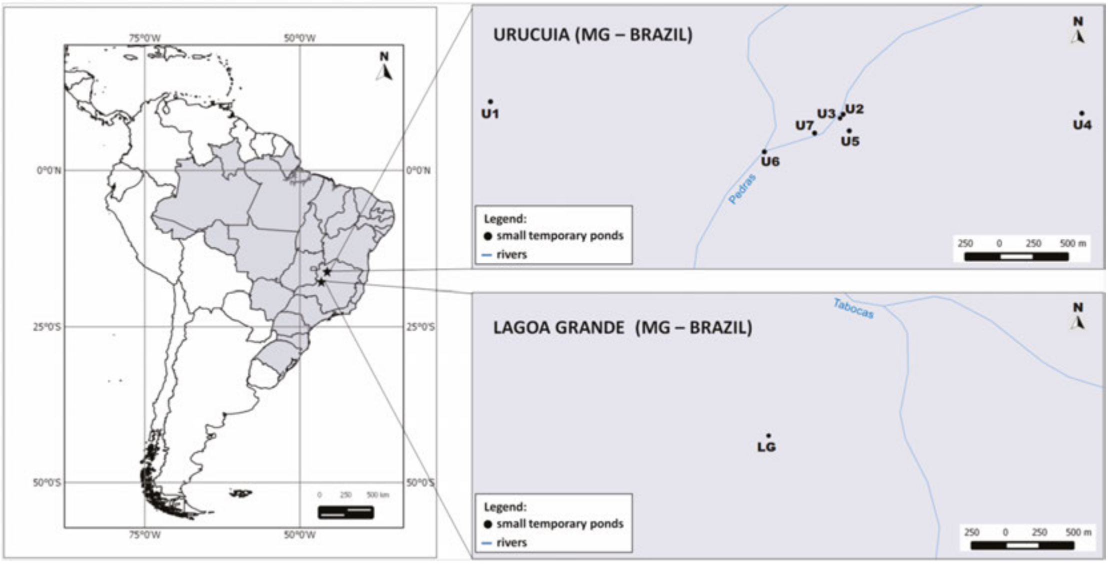


Figure 1. Location map of the study areas, indicating seven temporary ponds in the city of Urucuia (U1 to U7) and one in the city of Lagoa Grande (LG), all in the State of Minas Gerais, Brazil. *Mapa de localização das áreas de estudo, indicando sete lagoas temporárias na cidade de Urucuia (U1 a U7) e uma na cidade de Lagoa Grande (LG), todas no Estado de Minas Gerais, Brasil.*

in the functional aspects of communities is fundamental in understanding the ecological patterns that shape the different ecosystems (Petchey & Gaston, 2006). Therefore, the aim of the present study is to describe the zooplankton community structure from tropical temporary aquatic ecosystems during a flood periods, under taxonomic and functional approaches. We can raise the hypothesis that the presence of anostraceans, generalistic supension feeders, as strong competitors, as well as the extreme environmental characteristics of these kind of shallow temporary ecosystems, imply in low richness of zooplanktonic species, specially big filter feeders, and species have strategies to avoid overlap of niches.

METHODS

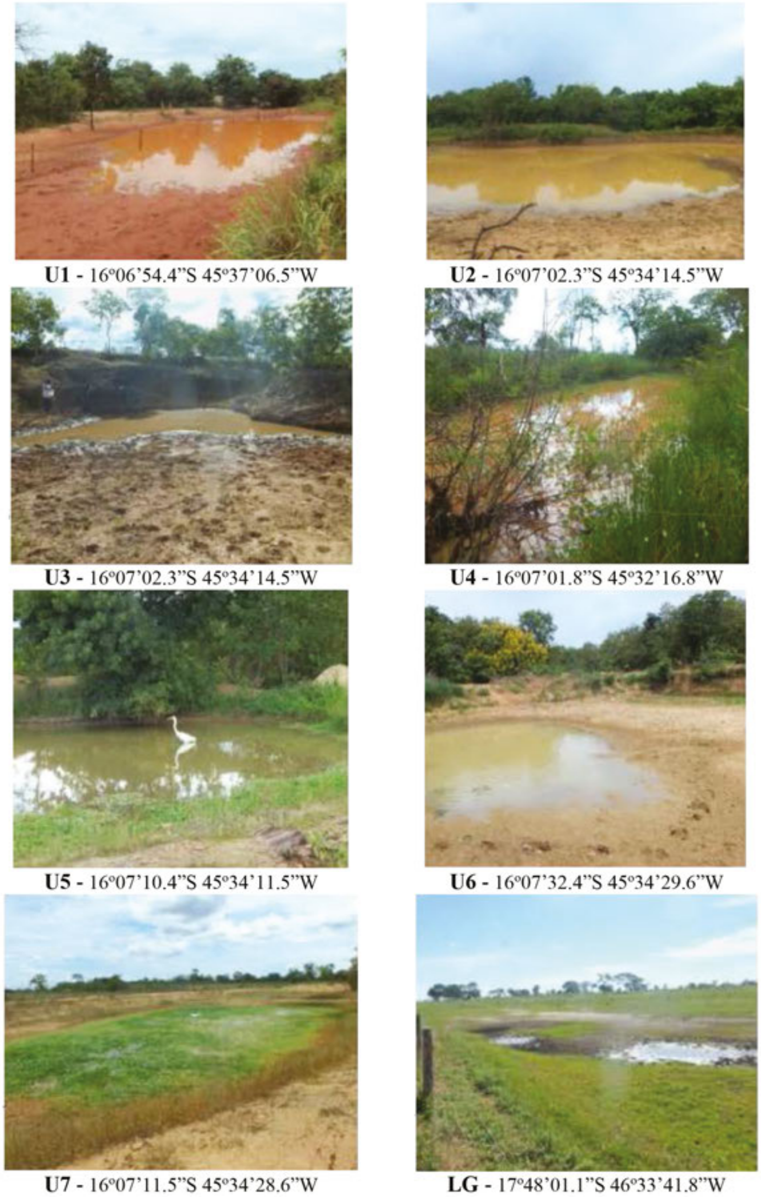
Study Area

Eight temporary ponds were sampled in the state of Minas Gerais (MG), Brazil: seven located in the municipality of Urucuia (U1 to U7) and one in the municipality of Lagoa Grande (LG) (Fig. 1) between January 31 and February 2, 2017. Figure 2 shows an overview of the studied environments and their geographic coordinates. These ponds usually flood between November and March, which is the period of greater precipitation in the northwest of MG. In this period, rainfall is usually around 600mm, according to the average of the three years before the sampling period of this study (Embrapa, 2013; Simge, 2015). Temporary ponds from this region are very shallow, even with maximum rainfall, rarely exceeding 1.0 m of profundity (Passos 2012; 2017).

To characterize the sampled ecosystems, the following limnological variables were measured: depth and transparency of the water column (Secchi disk), pH (pH meter Analion PM608), conductivity (conductivity analyzer C708), concentrations of total nitrogen - N (Valderrama, 1981), total phosphorus – P (Mackereth, 1978), suspended solids (total, organic and inorganic) (Cole, 1975) and chlorophyll *a* (Golterman *et al.*, 1978). Samples were taken in triplicate at one point in the middle of each pond.

Zooplanktonic Community

Zooplankton was collected using a 68 µm-mesh plankton net (20 cm-diameter) in open and central areas of the ponds. Only in the LG pond, which was very shallow, was water collected using a bucket (20 L) and then concentrated through the plankton net. The volume of filtered water in each sample was approximately 100 L and was calculated based on the distance covered by the net and its radius (cylinder volume formula). Collection procedures were performed 3 times in each pond and samples were then fixed in 4 % formaldehyde. Specimens at species level



U1 - 16º06’54.4”S 45º37’06.5”W

U2 - 16º07’02.3”S 45º34’14.5”W

U3 - 16º07’02.3”S 45º34’14.5”W

U4 - 16º07’01.8”S 45º32’16.8”W

U5 - 16º07’10.4”S 45º34’11.5”W

U6 - 16º07’32.4”S 45º34’29.6”W

U7 - 16º07’11.5”S 45º34’28.6”W

LG - 17º48’01.1”S 46º33’41.8”W

Figure 2. Overview of the studied temporary ponds, with the designation codes and geographical coordinates of each one. Ponds denominated U1 to U7 are located in the city of Urucuia, while the pond denominated LG is located in the city of Lagoa Grande, all in the State of Minas Gerais, Brazil. *Visão geral das lagoas temporárias estudadas, acompanhadas dos códigos de denominação e coordenadas geográficas de cada uma. As lagoas denominadas de U1 a U7 são localizadas na cidade de Urucuia, enquanto que a lagoa denominada LG está localizada na cidade de Lagoa Grande, todas no Estado de Minas Gerais, Brasil.*

were identified using a specialized bibliography. For qualitative and quantitative analyses, a stereoscopic microscope Leica® model MZ6 with magnification of up to 50 times and a Zeiss® optical microscope were used with a magnification up to 1000 times, both with a micrometered eyepiece. To estimate the relative abundances (population density expressed in individuals m^{-3}), individuals of sub-samples were counted in a Sedgewick-Rafter chamber (Rotifera and protozoans), under an optical microscope using grid acrylic plaques (Anostraca, Cladocera and Copepoda), under a stereoscopic microscope. Sub-samples were counted until no statistical variation was observed or no new species were found. In addition, the remaining material was examined at a smaller magnification to search for larger and less abundant taxa.

The length was also measured for each species and the biomass of crustaceans and rotifers were calculated from published regression equations. For rotifers, biomass was estimated from the biovolume calculated by means of specific mathematical formulas corresponding to the geometric forms that most resemble individuals (Ruttner-Kolisko, 1977). Thirty individuals of each species were measured under an optical microscope or, for those species that did not have this number; all individuals in the sample were measured. The biovolume was converted to wet weight considering the specific density of 1.0 ($1\ \mu m^3 = 10^{-6}\ \mu g$ wet weight) and then the correction factor 0.1 was used to convert wet weight to dry weight (Bottrell *et al.*, 1976). For Cladocera and Copepoda, biomass was determined by linear regression relating body length **- L** (mm) and weight **- W** (μg), that is, $\mathbf{Ln\ W = Ln\ a + b\ Ln\ L}$, where **Ln a** is 1.7512 for Cladocera and 1.9526 for Copepoda and **b** is 2.6530 for Cladocera and 2.3990 for Copepoda (Bottrell *et al.*, 1976).

Functional Diversity

Based on the literature (Barnett *et al.*, 2007, 2013; Sodré, 2014; Silva, 2015), eight functional traits were selected for all zooplanktonic species present in the studied ecosystems: (1) body length; (2) trophic group (herbivores, carnivores, omnivores, omnivores/carnivores and omnivores/herbivores); (3) feeding type (scrapers, suspension feeders, raptorial, sucking, aspiration and pseudopods); (4) type of reproduction (sexual or asexual); (5) food size range; (6) habitat (pelagic ou littoral); (7) number of reproductions per flood period (one or more); (8) duration of embryonic development.

Regarding the trophic group, we consider the predominant feeding of each species, although in many of them there may be some flexibility in the type of food ingested, for example, some herbivores can also feed on bacteria; we considered omnivorous species that do not have a restricted diet, having two or more types of food preference. Based on Hopp & Maier (2005), for Copepoda Cyclopoida, we consider the groups omnivores/-carnivores and omnivores/herbivores in order to contemplate young (omnivores) and adults (carnivores or herbivores)

Concerning the feeding type, scrapers were considered those that capture particles associated with any type of substrate; suspension feeders generate water flow in the search for food, capturing particles that are suspended (active search and passive capture); raptorial are the predators (both searching and capturing food are active); suckers are those who catch and suck on algae or small animals by means of an eversible mastax; aspiration to those that catch the prey due to aspiration created by sudden mastax dilatation; and those that use pseudopods to capture food (amoeboid protozoa). For the type of reproduction, we considered sexual or asexual.

In order to establish the size ranges of the food particles, we used the planktonic group sizes presented by Kalff (2002). For this study, we established five food size ranges: 0.2-10 μm; 10-15 μm; 15-40 μm; 40-100 μm; 60-300 μm. We considered the predominant feeding interval for each species as animal feed is not restricted to the established size classes. Femtoplankton (virus and bacteria < 0.2 μm) and nanoplankton (the smallest phytoplankton, ~0.5 to 2 μm) are food sources for Protozoa and Rotifera, although they may also be part of the feeding of filtering crustaceans such as daphnids. Nanoplankton (from 2 to 30 μm; often flagellated phytoplankton) is the main food source for micro and macrozooplankton, and small microplankton (30 to 70 μm) food for macrozooplankton. Microplankton (> 70 µm) and mesoplankton (200-20 000 µm; large cells and colonies) are food sources for macrozooplankton and pelagic or benthic omnivores, as well as protozoa and animals of the micro and macrozooplankton. Various studies have provided us with a basis for classifying animals in different food ranges (Burn, 1968; Pourriot, 1977; Ferguson *et al.*, 1982; Schoenberg & Carlson, 1984; Infante & Edmondson 1985; Knoechel & Holtby, 1986; Bodgan and Gilbert, 1987; Chaoruangrit *et al.*, 2017; Bern, 1994; DeMott, 1995; Pagano, 2008; Hopp & Maier, 2005).

Regarding the habitat, although the studied ponds were very small and shallow, which makes it difficult to separate the species, the classification took into consideration the species that usually live in open waters (pelagic) or those that usually associate with some type of substrate, such as macrophytes, filamentous algae or sediments (littoral), according to the literature (Barnett *et al.*, 2007; 2013).

For the duration of embryonic development (tED), we considered mean values found in the literature (25 to 27 ºC) for the same species from this study or as close as possible. Although protozoa do not have an embryonic development, we include them in the lowest category, since the time it takes to generate a new individual by bipartition is very short. We obtained the tED data from the following literature: Hutchinson 1957; Edmondson, 1960, 1965, 1974; Gras & St-Jean, 1969, 1976, 1983; Edmondson & Winberg, 1971; Herzig, 1983; Yúfera, 1987; Vijverberg, 1989; Smirnov, 1992; Hardy & Duncan, 1994; Wyngaard *et al.*, 1994; Ritzler, 1995; Irvine & Waya, 1999; Kepeler, 1999; Melão, 1997, 1999; Maia-Barbosa, 2000; Dumont & Negrea, 2002; Kalff 2002; Hausmann *et al.*, 2003; Melão & Rocha, 2004; Choueri *et al.*, 2007; Santos-Wisniewski & Rocha, 2007; Ma *et al.*, 2010; Brito *et al.*, 2016.

Data analysis

Qualitative and quantitative analysis of zooplankton were made according to CETESB (2000) and Bicudo & Bicudo (2004). In order to investigate the correlations between environmental variables and the numerical density of zooplankton community of the eight temporary ponds studied, a canonical correspondence analysis (CCA) was performed, by means of the software free R 3.4.2 (R Development Core Team, 2017), and a Pearson correlation coefficient analysis was also performed, where the standard correlation coefficients between the scores of the samples derived from the species density data and the scores of the samples obtained from linear combinations of the environmental variables are presented The Pearson correlation coefficient measures the degree of correlation between two metric scale variables, as well as the direction of the same; if it is a positive correlation (directly proportional = + 1), negative (inversely proportional = -1) or null (absence of correlation, = 0). The frequency of occurrence (%) of each species was calculated considering data from all ponds by the formula F = 100 * Pa / P (Pa = number of samples with a given species; P = total number of samples) (Dajoz, 1983).

Based on the data from the functional traits of each species, the definition of functional groups was performed by multivariate cluster analysis (Pla *et al.*, 2012). A dissimilarity analysis was performed using the Euclidean method to generate a dendrogram, using the Ward method, which produces better-defined groupings than other methods As the clustering algorithms do not determine an ideal number of groups, from the dendrogram it was possible to observe changes in the levels of dissimilarity for the successive stages of the clustering. The straight lines of the pooled individuals are of height corresponding to the level at which the individuals are considered similar. The software used was the free R 3.4.2 (R Development Core Team, 2017), using the FD package (Laliberté and Legendre, 2010; Laliberté *et al.*, 2014). In order to verify the existence of a correlation between the environmental parameters and the functional groups, canonical correspondence analysis (CCA) was performed using the free software R 3.4.2 (R Development Core Team, 2017). The level of significance between the environmental and biotic parameters was also analyzed using the Monte Carlo test, establishing a $p \leq 0.5$.

The functional dispersion index (FDis) was calculated, which ranges from 0 to 1.0, and reflects how the species are distributed in the environment, also relating the number of functional traits, the number of species and their respective abundances (Cianciaruso, 2009; Sodré, 2014; Silva, 2015). This index was calculated from two matrices: species x functional traits and species x relative abundances using the FD package of the free software R 3.4.2.

To analyze taxonomic diversity, Shannon Diversity (H') index were calculated through the Vegan package of free software R 3.4.2.

RESULTS

Limnological variables measurements recorded in the studied temporary ponds (Table 1) indicate that, in general, water transparency was very low; pH was slightly acid to neutral; there was low conductivity; the concentrations of total nutrients (N and P) were very high; the concentrations of suspended solids were variable (the largest portion was inorganic); and chlorophyll *a* concentrations were very low.

The zooplankton community identified in the eight sampled ponds comprises Rotifera species (11); Cladocera (7); Copepoda Cyclopoida (3); testate amoeba (3) and Anostraca (2) (Table 2). Anostraca was frequent in seven out of the eight sampled ponds; only in pond U7 there were no such large Branchiopoda. Among the cladocerans, the most frequent species was *Moina minuta*, followed by *Simocephalus latirostris*. Copepoda Cyclopoida and Rotifera species were less frequent (Table 2). Considering each pond, richness was very low (3 species in LG; 5 species in U6; 6 species in U1, U3, U4 and U5; 8 species in U2; and 13 species in U7). Concerning zooplankton biomass of all ponds, Anostraca was the most representative group, followed by Copepoda, Protozoa, Cladocera and Rotifera. Pond U6 had the highest total biomass, followed by LG and U4 (Table 2).

The first two axes of CCA applied to limnological variables and diversity of zooplankton community explained 91.5 % of the data (Fig. 3). The most effective environmental variables in the axes were: T.S; T.P; T.N and conductivity. Anostraca was strongly related to P, N and total solids, while Cladocera was slightly correlated with N and P. Copepoda and protozoa were very poorly related to conductivity, transparency and depth. The least effective environmental variables in the axes were depth and pH, the latter being weakly related to the Rotifera.

The Pearson correlation coefficient between Anostraca and total solids, total phosphorus and total nitrogen was 0.1603; 0.7561 and 0.7551, respectively. Total solids are strongly related to

Table 1. Values of limnological variables of eight temporary ponds located in the State of Minas Gerais, Brazil. MD = mean depth; Tp = transparency (Secchi disk); Cond. = conductivity; TN = total nitrogen; TP = total phosphorus; TS = total suspended solids; IM = inorganic fraction of suspended solids; OM = organic fraction of suspended solids; Chl = chlorophyll *a*. *Valores de variáveis limnológicas de oito lagoas temporárias localizadas no estado de Minas Gerais, Brasil. MD = profundidade média; Tp = transparência (disco de Secchi); Cond. = condutividade; TN = nitrogênio total; TP = fósforo total; TS = sólidos suspensos totais; IM = fração inorgânica de sólidos em suspensão; OM = fração orgânica de sólidos em suspensão; Chl = clorofila* a.

	MD cm	TP cm	pH	Condut. µS/cm	TN µg/L	TP µg/L	TS mg/L	IM mg/L	OM mg/L	Chl µg/L
U1	25	5	5.9	18.9	2233 ± 66.7	133.3 ± 5.0	225.3 ± 9.3	198.7 ± 2.8	26.6 ± 1.5	0.025 ± 0.004
U2	30	5	7.3	58.4	2300 ± 33.3	92.5 ± 0.8	136.5 ± 6.3	122.3 ± 2.9	14.2 ± 1.9	0.067 ± 0.007
U3	35	5	6.6	42.7	4450 ± 50.0	110.0 ± 1.7	47.5 ± 2.0	39.5 ± 1.4	8.0 ± 1.0	0.018 ± 0.004
U4	15	5	7.2	53.3	4733 ± 33.3	147.5 ± 4.2	59.9 ± 1.8	52.3 ± 0.7	7.6 ± 0.3	0.123 ± 0.012
U5	20	7	6.6	39.1	2216 ± 50.0	88.3 ± 3.3	19.9 ± 0.8	15.4 ± 2.2	4.5 ± 1.1	0.010 ± 0.004
U6	30	5	6.6	32.5	2083 ± 50.0	90.8 ± 0.8	37.7 ± 1.9	31.2 ± 0.9	6.5 ± 0.5	0.037 ± 0.005
U7	10	10	6.7	33.8	3000 ± 33.3	110.8 ± 0.8	39.4 ± 2.2	25.0 ± 1.0	14.4 ± 0.6	0.052 ± 0.007
LG	8	8	6.6	30.8	*	1714.2 ± 4.2	833.9 ± 4.3	707.5 ± 2.6	126.4 ± 2.0	0.019 ± 0.005

Table 2. Numerical densities (ind./m^3), biomass – numbers in brackets (µgDW/m^3), occurrence frequency (OF %) and frequency (Freq.) classification of species of the zooplankton community from eight temporary ponds located in the State of Minas Gerais (Urucuia – U1 to U7, and Lagoa Grande – LG), Brazil. VF = very common; F = frequent; LF = less frequent; S = sporadic. *Densidades numéricas (ind./m^3), biomassa – números entre parênteses (µgPS/m^3), frequências de ocorrência (OF %) e classificação da frequência (Freq.) das espécies da comunidade zooplanctônica de oito lagoas temporárias localizadas no Estado de Minas Gerais (Urucuia – U1 a U7, e Lagoa Grande – LG), Brasil. VF = muito frequente; F = frequente; LF = pouco frequente; S = esporádica.*

	U1	U2	U3	U4	U5	U6	U7	LG	OF%	Freq.
Anostraca										
Dendrocephalus brasiliensis		145 (27 405)	86 (43 000)	108 (183 600)	45 (13 500)	117 (257 000)			75,0	F
Dendrocephalus thieryi	58 (106 140)							230 (391 000)	12,5	S
Cladocera										
Alona glabra							250 (328)		12,5	S
Ceriodaphnia cornuta cornuta							700 (906)		12,5	S
Ceriodaphnia cornuta righaudi				2 (6)					12,5	S
Ilyocryptus spnifer					42 (72)		500 (818)		25,0	LF
Macrothrix squamosa							300 (460)		12,5	S
Moina minuta	840 (941)	157 (179)	900 (1117)	48 (58)	1000 (1118)		4719 (5261)	3200 (3569)	87,5	VF
Simocephalus latirostris		57 (213)	20 (75)		42 (157)		990 (3678)		50,0	F
Copepoda										
Cyclopoida										
Mesocyclops longisetus				49,3 (54)					12,5	S
Thermocyclops decipiens		139 (278)	330 (769)				820 (1862)		37,5	LF
Thermocyclops minutus	230 (390)	2899 (3858)							25,0	LF
Young (nauplii/copepodite)					169 000 (73 975)	48 750 (49 824)		160 (187)	37,5	LF
Rotifera										
Asplanchna sieboldi				5 (8,9)			100 (194)		25,0	LF
Brachionus angularis						2250 (450)			12,5	S
Brachionus calyciflorus		157 (31,4)							12,5	S
Lecane bulla					1250 (329,5)				12,5	S
Lecane curvicornis	340 (70,4)						500 (103,5)		25,0	LF
Lecane furcata							230 (36,7)		12,5	LF
Lecane lunaris	800 (166)		1200 (249)				1900 (393)		37,5	LF
Lecane papuana		1600 (192)							12,5	S
Plathionus patulus		900 (435)	1000 (483)				500 (242)		37,5	LF
Polyarthra aff vulgaris	507 (312)						702 (432)		25,0	LF
Bdelloidea				2 (1,8)		58 500 (11 285)		4000 (880)	37,5	LF
Protozoa										
Arcella mitrata						750 (61 425)			12,5	S
Difflugia corona					3750 (9375)				12,5	S
Difflugia oblonga						750 (20 873)			12,5	S
Total Density	**2775**	**6054**	**3536**	**214**	**175 129**	**111 117**	**12 211**	**7590**		
Total Biomass	**108 019**	**32 591**	**45 693**	**183 728**	**98 526**	**400 857**	**14 714**	**395 636**		

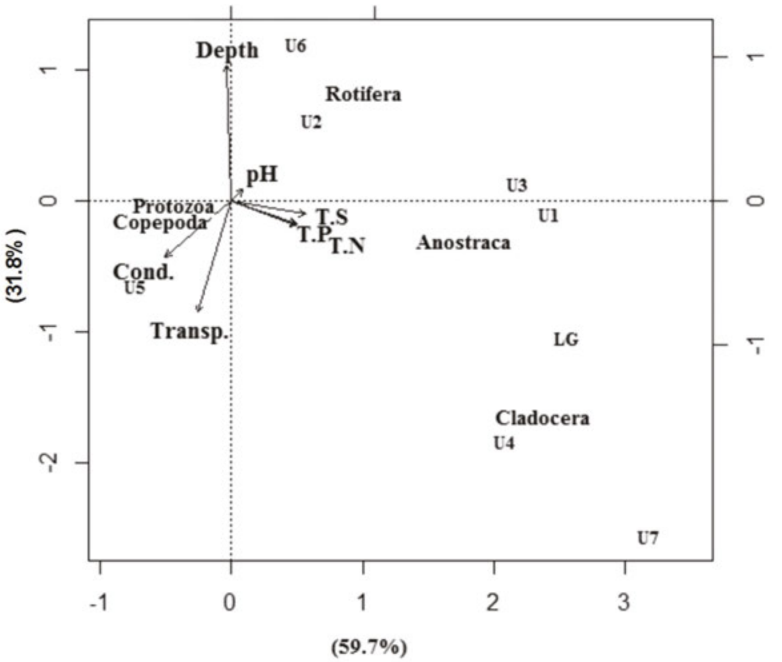


Figure 3. Canonical Correspondence Analysis (CCA) applied to limnological parameters and to the diversity of zooplankton groups from eight temporary ponds located in the State of Minas Gerais (Urucuia – U1 to U7, and Lagoa Grande - LG), Brazil. TS = total suspended solids; TN = total nitrogen; TP = total phosphorus; Transp. = transparency; Cond = conductivity. *Análise de Correspondência Canônica (CCA) aplicada aos parâmetros limnológicos e à diversidade de grupos do zooplâncton de oito lagoas temporárias localizadas no Estado de Minas Gerais (Urucuia – U1 a U7, e Lagoa Grande – LG), Brasil. TS = sólidos suspensos totais; TN = nitrogênio total; TP = fósforo total; Transp. = transparência; Cond = condutividade.*

organic and inorganic matter, being this correlation of 0.9074 and 0.9988. For Cladocera, the correlations were -0.2320 with total solids, 0.2352 with total phosphorus and 0.2300 with total nitrogen. For Copepoda and Protozoa, the correlation coefficient was close to zero. For Copepoda, the variables closest to presenting a tendency to correlation were, transparency (0.0813) and depth (0.0366), as well as for protozoa, transparency (0.0517) and depth (0.0685). Rotifera had a low correlation coefficient (0.3200) with depth, and a relation with negative tendency for the other variables (Table S1, see supplementary information available at http://www.limnetica.net/en/limnetica)

Although cladocerans and rotifers may have sexual or asexual reproduction depending on environmental pressures, in the samples of this study, sexual females (ephippial) were absent for all species, therefore they had only asexual reproduction.

The dendrogram generated by multivariate cluster analysis with the distances between zooplankton species (Fig. 4), grouping them according eight functional traits (Table 3), resulting in the following five functional groups. G1: large pelagic herbivores, suspension feeders, ingesting food particles from 60 to 300µm, the unique group with only one sexual reproduction per flood period, and mean duration of embryonic development (tED) of 1.4 days (*D. brasiliensis* and *D. thieryi*). G2: small littoral omnivores that capture small food particles (0.2-10 µm) with pseudopods, with asexual reproduction by bipartition (many division cycles per flood period) and low tED (< 0.2 days) (testated amoeba species). G3: small pelagic or littoral herbivores, mostly ciliate suspensivorous, ingesting small food particles (10-15 µm), many asexual reproduction cycles per flood period and fast embryonic development, from 0.6 to 1.0 days (all rotifers except *Asplanchna sieboldi*). G4: medium pelagic or

Table 3. Functional traits used for species of the zooplankton community of eight temporary ponds located in the State of Minas Gerais, Brazil. BL = body length (mm); TG = trophic group (H = herbivore; O = omnivore; C = carnivore; O/C = omnivore/ carnivore; O/H = omnivore/ herbivore); FH = food habit (SF = suspension feeders; Scr = scraper; Rap = raptorial; Suc = sucking; Asp. = aspiration; P = pseudopodia); R = reproduction (Ax = asexual; Sx = sexual); FSR = food size range; Hab = Habitat (Lit = littoral; Pel = pelagic); NR/F = number of reproductions per flood period (R1 = only one reproduction; R+ = several reproductions); tED = duration of embryonic development in days. *Traços funcionais utilizados para as espécies da comunidade zooplanctônica de oito lagoas temporárias localizadas no Estado de Minas Gerais, Brasil. BL = comprimento do corpo (mm); TG = grupo trófico (H = herbívoro; O = onívoro; C = carnívoro; O/C = onívoro/carnívoro; O/H = onívoro/herbívoro); FH = hábito alimentar (SF = suspensívoro; Scr = raspador; Rap = raptorial; Suc = sugador; Asp. = aspirador; P = pseudópodes); R = reprodução (Ax = assexuada; Sx = sexuada); FSR = intervalo de tamanho do alimento; Hab = Habitat (Lit = litoral; Pel = pelágico); NR/F = número de reproduções por período de cheia (R1 = apenas uma reprodução; R+ = várias reproduções; tED = duração do desenvolvimento embrionário em dias.*

	FUNCTIONAL TRAITS							
	BL	**TG**	**FH**	**R**	**FSR**	**Hab**	**NR/F**	**tED**
Anostraca								
Dendrocephalus brasiliensis	17.02	H	SF	Sx	60-300 µm	Pel	R1	1.4
Dendrocephalus thieryi	21.00	H	SF	Sx	60-300 µm	Pel	R1	1.4
Cladocera								
Alona glabra	0.29	H	Scr	Ax	10-15 µm	Lit	R+	1.6
C. cornuta cornuta	0.49	H	SF	Ax	15-40 µm	Pel	R+	1.7
C. cornuta righaudi	0.54	H	SF	Ax	15-40 µm	Pel	R+	1.7
Ilyocryptus spnifer	0.62	H	Scr	Ax	15-40 µm	Lit	R+	2.5
Macrothrix squamosa	0.58	H	Scr	Ax	15-40 µm	Lit	R+	1.7
Moina minuta	0.42	H	SF	Ax	15-40 µm	Pel	R+	1.4
Simocephalus latirostris	1.40	H	SF	Ax	40-100 µm	Pel	R+	1.8
Copepoda Cyclopoida								
Mesocyclops longisetus	1.08	OC	Rap	Sx	40-100 µm	Lit	R+	1.8
Thermocyclops decipiens	0.97	OH	Rap	Sx	15-40 µm	Lit	R+	1.9
Thermocyclops minutus	0.72	OH	Rap	Sx	15-40 µm	Lit	R+	1.9
Rotifera								
Asplanchna sieboldi	0.68	C	Asp	Ax	40-100µm	Pel	R+	1.0
Brachionus angularis	0.12	H	SC	Ax	10-15 µm	Pel	R+	0.9
Brachionus calyciflorus	0.32	H	SC	Ax	10-15 µm	Pel	R+	0.9
Lecane bulla	0.13	H	SC	Ax	10-15 µm	Lit	R+	0.6
Lecane curvicornis	0.12	H	SC	Ax	10-15 µm	Lit	R+	0.6
Lecane furcata	0.11	H	SC	Ax	10-15 µm	Lit	R+	0.6
Lecane lunaris	0.12	H	SC	Ax	10-15 µm	Lit	R+	0.6
Lecane papuana	0.10	H	SC	Ax	10-15 µm	Lit	R+	0.6
Plathionus patulus	0.13	H	SC	Ax	10-15 µm	Pel	R+	1.0
Polyarthra aff vulgaris	0.16	H	Suc	Ax	10-15 µm	Pel	R+	0.9
Bdelloidea	0.16	H	SC	Ax	10-15 µm	Lit	R+	1.0
Protozoa								
Arcella mitrata	0.16	O	P	Ax	0.2-10 µm	Lit	R+	< 0.2
Difflugia corona	0.19	O	P	Ax	0.2-10 µm	Lit	R+	< 0.2
Difflugia oblonga	0.21	O	P	Ax	0.2-10 µm	Lit	R+	< 0.2

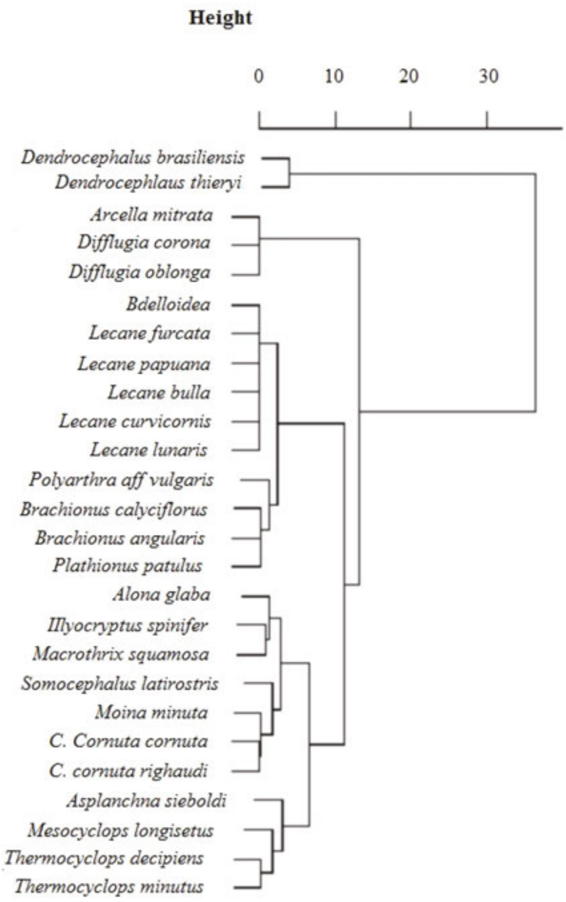


Figure 4. Dendrogram generated by multivariate Cluster analysis with the distances between zooplankton species, and grouping them according eight functional traits. *Dendrograma gerado por análise multivariada de Cluster com as distâncias entre espécies zooplanctônicas, agrupando-as de acordo com oito traços funcionais.*

littoral herbivores, suspensivorous or scrapers, ingesting food particles from 10 to 100 µm, with many asexual reproductive cycles per flood period, and tED from 1.4 to 2.5 days (all Cladocera). G5: pelagic or littoral raptorial predators (predominantly omnivorous or carnivorous), ingesting food particles from 15 to 100µm, with many asexual or sexual reproduction cycles per flood period, and tED from 1.0 to 1.9 days (all Copepoda and the rotifer *Asplanchna sieboldi*).

From data of the relative abundance of the five functional groups in each temporary pond (Table 4), a CCA relating this abundance to the limnological parameters (Fig. 5) shows that 80.8 % of the data was explained by the first two axes, where G3 was correlated to ponds' mean depth; G4 was related to water transparency, total suspended solids, total P and N; G5 was correlated to conductivity and pH; and G1 and G2 did not present direct correlation with any analyzed variables.

Values of two indexes, Shannon Diversity (H') and Functional Diversity (FDis), calculated for the zooplankton community of the studied ponds are, respectively: 1.04/0.18 for U1; 1.03/0.31 for U2; 0.95/0.19 for U3; 1.43/0.38 for U4; 1.28/0.28 for U5; 1.30/0.33 for U6; 1.36/0.30 for U7; and 1.03/0.15 for LG. It can be observed H' ranging from 0.95 to 1.43 and low values of FDis (0.15 to 0.38). Ponds also presented low values of species richness (3 to 13 species).

DISCUSSION

Characteristics of the studied ecosystems

The results of this study indicate that because all the sampled ephemeral ecosystems are small, very shallow (mean depth from 8 to 30 cm) and are rain-fed ponds isolated from another source of water, there is a high suspension of sediment particles (which causes low transparency - 2 a 10 cm), especially the inorganic ones (from 63.5 % in U7 to 89.6 % in U2). Most of the studied ponds have little or no vegetation in their surroundings, which may be related to high amounts of suspended solids and inorganic matter, which was also observed by Sahuquillo *et al.* (2012). Inorganic particles adsorb phosphorous reducing its bioavailavility in water. Due to this, although we found high amounts of total phosphorus (and also total nitrogen), comparable to eutrophic lakes, planktonic chlorophyll *a* was very low and not correlated with total nutrients, probably due to low light penetration in these environments, which limits the development of the phytoplanktonic community. The organic fraction of suspended material was low in all ponds, but proportionally higher in those covered by grasses (U7; LG) or with more vegetation in its surroundings (U5). This probably also applies to the sediment due to the short hydroperiod of ponds (about three months per year) since desiccation

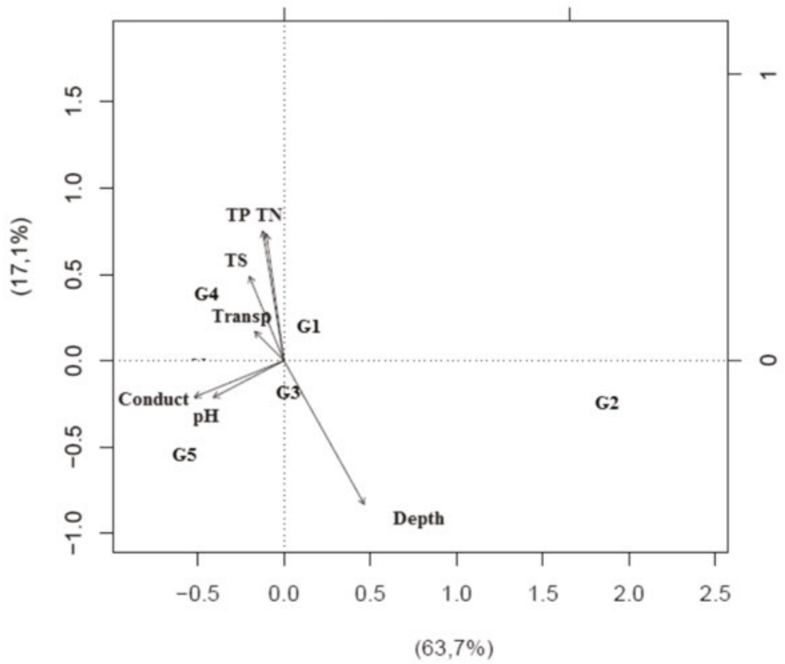


Figure 5. Canonical Correspondence Analysis (CCA) applied to limnological parameters and relative abundances of five functional groups (G1 to G5) of the zooplankton community from eight temporary ponds located in the State of Minas Gerais, Brazil. TS = total suspended solids; TN = total nitrogen; TP = total phosphorus; Transp. = transparency; Conduct = conductivity. *Análise de Correspondência Canônica (CCA) aplicada aos parâmetros limnológicos e às abundâncias relativas de cinco grupos funcionais (G1 a G5) da comunidade zooplanctônica de oito lagoas temporárias localizadas no Estado de Minas Gerais, Brasil. TS = sólidos suspensos totais; TN = nitrogênio total; TP = fósforo total; Ttransp. = transparência; Conduc = condutividade.*

has important effects on biogeochemical transformations. A threshold of 5 % of organic matter in the sediment separates the temporary lagoons with a short hydroperiod of those with a long hydroperiod. The shallower the pond, the shorter the hydroperiod and the lower the organic matter that the sediment contains, the lesser the total nitrogen proportion with respect to total phosphorus (Sahuquillo *et al.*, 2012). These authors also state that an important loss of N occurs during desiccation through mineralization, ammonia volatilization and the sequential nitrification/denitrification pathway and that the increase of organic phosphorus mineralization in dry sediments does not represent a loss of P from the system, since it remains adsorbed to the soil, which also leads to a low TN/TP ratio. Despite this, all ponds studied here had high amounts of total nitrogen and a high proportion with respect to total phosphorus, at least in the water column. As these ponds are widely used in the region for cattle watering, which in addition to trampling suspending the sediment possibly contributes to increasing the nitrogen concentration in the water with their feces and urine. In addition, the high temperatures of these shallow environments may favor the processes of releasing nutrients from sediment.

The pH did not vary much between the studied ponds, ranging from slightly acid to neutral (5.9 to 7.3), close to the most continental water bodies whose pH ranges from 6.0 to 8.5 (Margalef, 1983; Kalff, 2002). The decomposition of the organic matter decreases the water pH. However, as the amount of organic matter in these environments is low, probably its pH is more related to geological characteristics and soil type sites. The ponds are located in a Brazilian savanna (“cerrado”) region, near the semi-arid region of northeast Brazil, where soils tend to be sandy with a higher acidity. Similarly, conductivity does not seem to be associated with the decomposition in these environments, since

Table 4. Relative abundance (%) of the functional groups (G1 to G5) defined for the zooplankton community of eight temporary ponds located in the State of Minas Gerais, Brazil. Ponds U1 to U7 are located in the city of Urucuia and LG, in the city of Lagoa Grande. *Abundância relativa (%) dos grupos funcionais (G1 a G5) definidos para a comunidade zooplanctônica de oito lagoas temporárias localizadas no Estado de Minas Gerais, Brasil. As lagoas U1 a U7 são localizadas na cidade de Urucuia e LG, na cidade de Lagoa Grande.*

	U1	U2	U3	U4	U5	U6	U7	LG
G1	16.67	12.50	16.67	16.67	16.67	20.00	0.00	33.33
G2	0.00	0.00	0.00	0.00	16.67	40.00	0.00	0.00
G3	50.00	37.50	33.33	16.67	16.67	40.00	38.45	33.33
G4	16.67	25.00	33.33	33.33	50.00	0.00	46.15	33.33
G5	16.67	25.00	16.67	33.33	0.00	0.00	15.40	0.00

values did not differ much between the ponds and were not very high, even in ponds covered by vegetation such as U7. Thus, conductivity should be more associated with the drainage basin phenomena, such as the nature of rocks and soil of the basin, the type of vegetation and the effects of rainwater leaching. Ions concentration in water is also strongly dependent on the temperature, which is quite high in these ponds, often exceeding 30 ºC. In fact, in tropical regions, conductivity of aquatic environments is more related to the geochemical characteristics of the region where they are located and to the climatic conditions (dry and rainy season) than with the trophic state, as in temperate regions, which have in general higher conductivity values (Esteves, 1988).

Therefore, temporary aquatic ecosystems have unique characteristics connected to dry periods and rainy seasons that influence the fluctuation of the water level and, as a consequence, have their physical and chemical characteristics altered (Maltchik *et al.*, 1999). Important factors such as rainfall and evaporation rates have a significant impact on the dynamics of these ecosystems (Tundisi & Matsumura Tundisi, 2008).

Zooplankton Community

Taxonomic diversity and biomass

In the present study, we found a very low diversity of the zooplankton community in all ponds studied. Species richness in each pond ranged from 4 to 13, and we found only 27 taxa considering all ponds, recorded among Anostraca, Cladocera, Copepoda, Rotifera and Protozoa. Other studies in Brazilian aquatic ecosystems recorded much higher zooplankton species richness both in temporary and in perennial environments (reservoirs and rivers). Passos (2012) found 20 to 23 taxa in three shallow temporary ponds in the municipality of Urucuia, Minas Gerais (near to the ponds of this study); Passos (2017) registered 68 taxa for a total of 25 shallow temporary ponds from Minas Gerais (municipalities of Urucuia, Lagoa Grande and Pirapora) studied during three consecutives years (2015 to 2017); Moreira *et al.* (2016) registered 29 taxa in a shallow high-elevation temporary pond in Minas Gerais; in São Paulo State, Melão (1997) found 28 taxa in a shallow permanent oligotrophic reservoir and Santos (2010) found richness ranging from 17 to 36 in three large big reservoirs of Tietê River (São Paulo); Silva (2015), in a review of studies done in large amazonic rivers, reported 143 species in the Madeira River and 129 species in the Xingu River. It is possible, however, that our results underestimate the real richness of zooplankton species in the studied ponds, since we did a single sampling campaign and resistance eggs of the species from such temporary ecosystems can hatch in different times (Passos, 2017). Even so, it seems possible to identify a pattern in the coexistence between zooplanktonic species. It seems clear that larger filtering animals, such as large cladocerans species, are absent in the presence of *Dendrocephalus* species or may coexist with them but with low population densities. Passos (2017), studying 25 temporary ponds of Minas Gerais, recorded the occurrence of Copepoda Calanoida in ponds with absence or small population density of *Dendrocephalus brasilienses*. On the other hand, smaller cladocerans seem to coexist well with these large Branchiopoda. The most successful Cladocera species in the sampled environments in the present study is *Moina minuta*, both in terms of frequency of occurrence and number of individuals. Passos (2017) also recorded the occurrence of this species in 100 % of the 25 environments of his study, in addition to presenting the highest numerical densities. Among the Copepoda, the Cyclopoida are predominant in the temporary ponds studied, particularly the species of *Thermocyclops*. Passos (2017) also recorded *Thermocyclops decipiens* and *T. minutus* as the most frequent. Rotifera species are more numerous, but *L. lunaris* and *Plathionus patulus* are some of the most frequent in these temporary environments, which was also found by Passos (2017).

Temporary ecosystems are generally small, shallow and isolated (Pérez-Bilbao *et al.*, 2015), which results in a relatively low biodiversity (Zacharias *et al.*, 2007; Crispim & Freitas, 2005). In a study of 29 temporary ponds in the UK, divided into permanent (which have well defined periods of flood and dry) and temporary semi-permanent (which exceptionally dry up and can spend years in flood periods), the authors found a greatly reduced number of species in the semi-permanent temporary ponds compared to the others (Collinson *et al.*, 1995). These authors state that temporary environments are ecosystems of rare species, with much endemism, and report that, in addition to harboring rare plants and amphibians, 25 % of endangered freshwater invertebrate species inhabit temporary waters. Thus, temporary aquatic ecosystems have a great evolutionary and ecological importance, housing communities which are adapted physiologically and that develop efficient and diversified mechanisms for their colonization, permanence and reproduction.

In the present work, we found two Anostraca species, both from the Thamnocephalidae family: *Dendrocephalus brasiliensis* (ponds U2 to U6) and *Dendrocephalus thieryi* (ponds U1 and LG). In Brazil, so far, *D. thieryi* has had an occurrence recorded only for the municipality of Buritizeiro, MG (Rabet, 2006); therefore a new record was made of this species in the present study. The populations of the sampled *Dendrocephalus* had homogeneous ages (male and female adults) as diapause eggs hatch all at the same time when the pond is filled during the rainy season (Lopes, 2007).

Rotifera had the highest species richness in the studied ponds, as occurs in most aquatic ecosystems, especially in tropical regions (Rocha *et al.*, 1995), which is justified by the high population growth rates of r-strategist species, and also adaptation to the periodic alterations of these ecosystems (Allan, 1976). The other groups had lower species richness and their frequency of occurrence was generally low. Moreover, most of these species were classified as sporadic and infrequent. *Moina minuta*, *D. brasiliensis*, and *Simocephalus latirostris* had a higher frequency and *M. minuta* was the most frequent species in all the ponds sampled. This is explained by the success in the colonization strategies of these species, mainly the production of dormant eggs that resist the desiccation periods (Passos, 2017).

Related to Protozoa, probably the number of species is underestimated as the collection and fixation methodologies used in this study were not targeted at such organisms, and certainly promoted the disruption of these unicellular individuals. Only the carapaces of testate amoeba remained, which were recorded in this study.

Comparing the numerical densities of zooplankton in the eight studied ponds, we observed that in U7, where *Dendrocephalus* species are absent, there is a significant increase of all species of Cladocera, especially *M. minuta*, *Simocephalus latirostris* and *Ceriodaphnia cornuta*, as well as adults of Copepoda Cyclopoida *Thermocyclops decipiens*. There is, therefore, clear evidence that Anostraca interfered in the species composition and in the population densities of the Cladocera, possibly due to the niche overlap. In addition, there are few large species of Cladocera, with a predominance of small species, able to feed on bacteria and debris, since phytoplankton is relatively scarce in these environments. A larger Cladocera, *Simocephalus latirostris*, occurs in higher densities only in U7, probably due to the presence of aquatic plants, which occurs only in this pond.

Generally, the initial phase of an environment is dominated by larger species, with strategies of rapid colonization; and the other species, k-strategists, tend to colonize it later (Vanschoenwinkel *et al.*, 2011). The Anostraca species are large and r-strategists and can be good competitors for resources as they are generalists, adapted to temporary ecosystems and fast hatching and, because of this, interfere in the population composition of the other community groups (Sarma & Nandini, 2002). The zooplankton species of the temporary environments, however, have several strategies to avoid the niche overlap. One of them is the time lag in the hatching of resistance eggs of different zooplankton species (Passos, 2017), indicating a partition of niches between them, which would minimize the competition for resources, leading to the successful coexistence of these species in these temporary ecosystems.

The competition in these temporary ponds is intense, since the environmental conditions do not favor food abundance, especially phytoplankton due to low penetration of light. In the CCA, it can be observed that Anostraca and Cladocera (in lesser degree) are favored by parameters such as suspended solids, total nitrogen and phosphorus, which are indirectly related to the availability of food in the water column, such as debris and bacteria; Copepoda were slightly favored by variables (conductivity and transparency) that indicate better conditions for phytoplankton growth, from which their young phases feed and also the adults´ prey.

In terms of biomass, *Dendrocephalus* species far exceeded the other groups in all ponds where they occur. In the absence of the Anostraca (pond U7), the cladocerans *M. minuta* and *S. latirostris* stand out for the high biomass, followed by the copepod *T. decipiens*. The increase of biomass of these species should be related to the greater availability of food particles, since there is no presence of the big anostracean competitors.

Since there are variations in the size of the species, and each one plays a different role in the ecosystem, biomass more effectively describes the zooplankton community (Melão & Rocha, 2004), providing a perception of the trophic chain, energy and the actual contribution of each species to ecological processes (Begon & Townsend, 2006). However, the real role of different species in ecosystems also depends on how efficient they are in renewing their biomass. Rotifers, for example, are small but have a high rate of renewal of their populations, that is, they have a high production/biomass ratio (P/B ratio). Other groups, such as Copepoda, for example, are larger and they have relatively high fecundity, but have a longer time of embryonic and post-embryonic development, which could result in lower population turnover rates compared to Cladocera or Rotifera. In the temporary ecosystems studied here, species that have higher population turnover rates have competitive advantages over others, since the hydroperiod is very short. These questions are very important in the ecology of zooplankton and completely unknown to Brazilian temporary ponds, and therefore more in-depth studies are needed.

Functional Diversity

Analyzing the functional diversity of the studied ponds indicated that the most determinant attributes to group the species were the trophic group and feeding habits. Although the attributes associated with reproduction were also important, food relationships were more decisive. In small environments, as specific as those sampled in this study, a certain convergence in the way of feeding is natural, especially due to scarcity or less variety of resources. In the sampled ponds, a predominance of species occurs with passive food capture, even if the search is active. Each type of feed has costs and benefits, and passive feeding allows for less energy expenditure, and a low risk of predator exposure. The size of food particles is also a criterion that can determine the grouping of species, and is directly related to food habits (Barnett *et al.*, 2007). Herbivores, which were found in half of the functional groups of this study, tend to feed on smaller particles, which generally fluctuate in the aquatic environment, favoring passive feeding, while carnivores tend to feed on larger particles as they spend more energy on active feeding activities. The size of the food particles, therefore, can determine patterns of food strategies (Litchman, 2013).

In CCA, the functional groups correlated with environmental variables favor greater food availability. The group of raptorial predators (G5), are favored by the presence of prey, which in turn are more correlated to the variables that favor the growth of bacteria or phytoplankton like transparency and nutrients; the filter-feeders (especially G4) are favored by variables like TP, TN, TS that favor the presence of food in the water column (especially bacteria and mixotrophic nanoflagellates), and by an increase in light penetration, which increases phytoplankton growth.

Type of reproduction is important in temporary aquatic ecosystems, where species need to invest in strategies to ensure their permanence considering so many environmental oscillations. Reproduction occurs asexually (by parthenogenesis) in the flood period, but the stress caused when the environment is drying induces the formation of resistance eggs through sexual reproduction. In this functional trait, we considered the reproduction predominant for each species according to our samples, although there may be changes depending on the environmental conditions. Reproduction and growth rates of zooplankton community are a reflection of the ways of allocating energy (Litchman, 2013). Investing in growth or reproduction, for example, can be a decisive choice for species. In the zooplankton community this trade-off in investment is very clear: investment in reproduction implies a reduced growth and reduced lifetime. On the contrary, investment in growth automatically implies less longevity. Rotifers, for example, allocate more energy to reproduction, and fail to invest in body size and longevity. In contrast, copepods have defined stages of development, with stages of maturation and greater longevity, which implies in a lower risk of predation (Huntley & Lopez, 1992). Moreover, concerning reproduction, a large number of offspring is directly related to a smaller body size of each individual of offspring, which results in the reduction in individual survival. This is a constant strategy for the permanence of species of the zooplankton community in the ecosystem (Litchman, 2013).

In the present study, the functional grouping of zooplankton community was very close to the taxonomic grouping, with the exception of G5 that grouped the three species of Copepoda Cyclopoida and one of Rotifera, mainly due to their similar eating habits (all are raptorial predators). This result may have been influenced by our choice of functional traits. However, it should be considered that the taxonomic classification of species is already based precisely on their morphological characteristics that are in some way related to the function performed by the species within the ecosystem (Silva, 2015). In general, natural selection tends to accentuate individual fitness, increasing the outcome of eating, survival and growth patterns within communities (Litchman, 2013). However, even if functional bias is similar to taxonomic grouping in many cases, it is important to know the functional attributes and also how they relate to the influences of the environment (Brasil & Huszar, 2011).

The functional diversity analyzed in this study for the zooplankton community was generally low (from 0.15 to 0.38 in an index that ranges from 0 to 1.0) for all environments sampled. This may indicate a dominance of few functional attributes, which may be the result of the strong action of environmental filters in the community structure and would determine the occurrence of species with more similar functional characters. Low functional diversity of zooplankton can therefore inform us that important processes that occur in the community structure can be influenced by the action of physical and/or chemical characteristics or environmental filters that act on the species, promoting a confluence of the functional characteristics. But on the other hand it is also possible that this occurs due a great variation within each zooplankton species in order to adapt to environmental conditions.

In a large spatial scale study with mammals of tropical ecosystems, Safi *et al.* (2011) identified low functional diversity in relation to the taxonomic one and affirm that this is a common pattern in tropical regions, suggesting that there is a high number of ecologically similar species due to a smaller evolutionary history between them, which also shows a low phylogenetic diversity. Considering the few studies on zooplankton in tropical regions, it is too early to say that this also applies to this community.

In fact, maybe zooplanktonic community is not as good as phytoplankton in studies of functional diversity. For phytoplankton, this kind of study is already well established (Brasil & Huszar, 2011), but it is not to zooplankton. As zooplankton presents several adaptation characters in individuals within the same species, it is difficult to choose traits that really differentiate them. For example, some cladocerans are larger in presence of invertebrate predators and smaller in the presence of visual predators (Caramujo *et al.*, 1997; Crispim & Boavida, 2001), so their length can change depending on the biological interactions; reproduction type (sexual or asexual) can also changes according environment conditions; fecundity also have great intraspecific differences, related, for example, to female size and food conditions (Vieira *et al.*, 2011); food source can also present great variability. Anyway, it is not easy choose the right functional traits, and the wrong choice can lead to misinterpretations of the results.

Thus, although studies related to functional diversity have advanced in recent years, as far as zooplankton communities are concerned, much remains to be done. A barrier that may account for such a deficiency would be the lack of basic studies on the biology of organisms, since with more detailed and accurate information; a greater number of functional traits could be used. There are many important traits for each species, but measuring them all is not possible for many reasons. Many of the traits to be studied have already been suggested by Litchman *et al.* (2013). Thus, better and broader measures of functional diversity would be possible, which would further explain the ecological functions of organisms and their relationship with ecosystems.

Using various ways to measure diversity can help assess evolutionary and ecological processes that act on diversity. The biological diversity of ecosystems has been investigated in recent decades with the help of many ecological theories of communities that address spatial and temporal patterns, such as the ecological succession theory (Gleason, 1927) and the theory of island biogeography (Macarthur & Wilson, 1967). In the theory of island biogeography, the research carried out by the authors is based on three observations: (1) Island communities are poorer in species than equivalent continental communities; (2) richness increases with island size; (3) richness decreases with increasing isolation of the island. Perhaps we can make an analogy here of the object of our study with islands as the temporary ponds studied in this research seem to follow the same premises of this theory: they are small and isolated ecosystems, which have a low diversity of species when compared to other ecosystems of continental waters.

Thus, the present study contributed to increasing knowledge on the taxonomic and functional diversity of the zooplankton community of temporary aquatic ecosystems in the Neotropics. Environmental conditions and very peculiar characteristics of these ephemeral ecosystems have resulted in low diversity indexes of the zooplankton community, where few species with similar ecological functions and similar strategies ensure establishment, permanence and reproduction in these environments, such as high reproduction capacity, rapid growth, short life cycles and type of feed. In addition, there was evidence that Anostraca species of the genus *Dendrocephalus* have an impact on the taxonomic composition and numerical density of the community, especially Cladocera.

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On the taxonomic status of some South American *Eucyclops* (Copepoda: Cyclopidae: Eucyclopinae)

Nancy Fabiola Mercado-Salas[1,2,*] and Eduardo Suárez-Morales[3]

[1] German Centre for Marine Biodiversity Research (DZMB), Senckenberg am Meer Wilhelmshaven, Südstrand 44, 26382 Wilhelmshaven, Germany.
[2] Centrum für Naturkunde - CeNak/Center of Natural History Hamburg, Universität Hamburg - Zoological Museum, Martin-Luther-King-Platz 3, 20146 Hamburg, Germany
[3] El Colegio de la Frontera Sur (ECOSUR), Unidad Chetumal. Av. Centenario Km. 5.5, Chetumal, Quintana Roo, 77014. Mexico.

[*] Corresponding author: nancy.mercado.salas@uni-hamburg.de

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ABSTRACT

On the taxonomic status of some South American *Eucyclops* (Copepoda: Cyclopoida: Eucyclopinae)

Among eucyclopinids freshwater copepods, the genus *Eucyclops* contains several species or species groups whose taxonomic status has not been properly determined and is in need of revision or redescription. Traditionally used meristic characters have recently been deemed insufficient for resolving these problems, particularly in respect of the South American taxa. New, upgraded descriptive standards and molecular tools have contributed greatly to the clarification of some of these cases in both Europe and the Americas. Based on examination of type specimens and museums' collections, and following currently accepted taxonomic standards, we were able to clarify the status of some problematic South American species of *Eucyclops*. We provide herein a redescription of three South American species (i.e., *E. pseudoensifer*, *E. breviramatus*, and *E. subciliatus*), each with a history of poorly understood affinities with other American *Eucyclops*. The nominal *Eucyclops neumani*, described by Pesta (1927) and originally described from Argentina, was known to contain two subspecies: *E. neumani neumani*, recorded in Argentina, Paraguay, and Brazil, and *E. neumani titicacae* (Kiefer, 1957), known only from Peru and Colombia. Based on our analysis, we conclude that *E. titicacae* is not a subspecies of *E. neumani*, but a separate species, distinguishable from its closest congeners. We also provide evidence showing that neither *E. pseudoensifer* nor *E. breviramatus* are members of the *E. serrulatus* species group.

Key words: freshwater crustaceans, taxonomy, morphology, zooplankton

RESUMEN

Revisión del estatus taxonómico de algunos* Eucyclops *(Copepoda, Cyclopoida, Eucyclopinae) de Sudamérica

Entre los copépodos euciclópinos, el género Eucyclops *contiene varias especies o grupos de especies cuya situación taxonómica no se ha resuelto adecuadamente y requieren revisión o redescripción. Los caracteres merísticos tradicionalmente utilizados han sido considerados como insuficientes para desentrañar estos problemas, particularmente entre especies sudamericanas. Los estándares descriptivos actuales y las herramientas moleculares han contribuido mucho a aclarar algunos de estos casos tanto en Europa como en América. Con base en el estudio de especímenes tipo, colecciones de museos y siguiendo estándares taxonómicos mejorados y actualizados, hemos podido aclarar el estado de algunas especies problemáticas de* Eucyclops *conocidas en Sudamérica. Presentamos redescripciones de tres especies sudamericanas (i.e.,* E. pseudoensifer, E. breviramatus, E. subciliatus*), cada una de ellas con una historia de afinidades poco claras respecto a otras especies americanas de* Eucyclops. *La especie nominal* Eucyclops neumani *(Pesta, 1927), originalmente descrita de Argentina, incluye dos subespecies,* E. neumani neumani, *encontrada en Argentina, Paraguay y Brasil, y* E. neumani titicacae *Kiefer, 1957, conocida solo en Perú y Colombia. Basándonos en nuestro análisis, llegamos a la conclusión de que* E. titicacae *no debe ser considerada una subespecie de* E. neumani*, sino una especie separada, distinguible de sus congéneres más cerca-*

nos. Derivado de la redescripción de estas tres especies sudamericanas, logramos aclarar su situación taxonómica y su afinidad con otros Eucyclops *americanos. También proporcionamos evidencias que muestran que tanto* E. pseudoensifer *como* E. breviramatus *no deben ser considerados como parte del grupo de especies de* E. serrulatus.

Palabras clave: *taxonomía, copépodos continentales,* Eucyclops

INTRODUCTION

The current knowledge of the genus *Eucyclops* in the Americas is based on more than 800 records obtained from the taxonomic work conducted over 100 years of biological sampling across the entire continent. Up to 36 nominal species of this genus have been recorded in the Americas, with most records coming from the United States, Mexico, Argentina, and Brazil (Reid, 1985; Mercado-Salas *et al.*, 2012; Mercado-Salas *et al.*, 2016). In South America, there are more than 150 extant records that correspond to 20 nominal species, Nine of these species are considered South American endemics (i.e., *E. silvestri* [Brian, 1927], *E. neumani* [Pesta, 1927], *E. neotropicus* [Kiefer, 1936], *E. titicacae* [Kiefer, 1957], *E. alticola* [Kiefer, 1957], *E. demacedoi* [Lindberg, 1957], *E. siolii* [Herbst, 1962], *E. subciliatus* [Dussart, 1984a], and *E. chilensis* Suárez-Morales & Walsh, 2009, with another 8 species reported in Central and North America (i.e., *Eucyclops elegans* [Herrick, 1884], *E. delachauxi* [Kiefer, 1925], *E. prionophorus* [Kiefer, 1931], *E. bondi* [Kiefer, 1934], *E. ensifer* [Kiefer, 1936], *E. leptacanthus* [Kiefer, 1956], *E. breviramatus* [Löffler, 1963], and *E. pseudoensifer* [Dussart, 1984]). A few nominal species of *Eucyclops* are known as cosmopolitan forms, presumably representing species-complexes: *E. serrulatus* (Fischer, 1851), *E. agilis* (synonym to *E. serrulatus*) and *E. speratus* (Lilljeborg, 1901) (see Kiefer, 1936; Lindberg, 1954; Dussart, 1984, 1984a; Reid, 1985; Dussart & Frutos, 1986; Defaye & Dussart, 1988; Santos & Andrade, 1997; Gaviria & Aranguren, 2007; Silva, 2008; De los Ríos *et al.*, 2010; Mercado-Salas *et al.*, 2012).

This genus is one of the most taxonomically challenging groups among the Cyclopidae because it contains several problematic taxa and species complexes with high intraspecific variability, a problem shared with other cyclopoid copepod genera like *Acanthocyclops* (Miracle *et al.*, 2013). Until recently, only a few species of *Eucyclops* have complete, upgraded descriptions. In the last 10 years, several valuable taxonomic advances have been published, especially in the Americas, to clarify the taxonomic status of these taxa. Some of these species are now defined more accurately because of the use of new morphological characters and the analysis of their geographic distributional patterns (Alekseev *et al.*, 2006; Defaye & Alekseev, 2011; Mercado-Salas *et al.*, 2012; Mercado-Salas & Suárez-Morales, 2014a, b; Mercado-Salas *et al.*, 2016). The key requirements for clarifying these closely related species of the American *Eucyclops* include: (1) the revision of the type specimens, (2) redescription of some species following upgraded standards, and (3) examination of additional material held in different zoological collections, especially at the Muséum National d'Histoire Naturelle (France), the Staatliches Museum für Naturkunde Karlsruhe (Germany), the National Museum of Natural History, Smithsonian Institution (USA), and the Natuurhistorisch Museum Wien (Austria). In previous works, we discussed the taxonomic position of some *Eucyclops* described by F. Kiefer (Karlsruhe) (see Mercado-Salas & Suárez-Morales, 2014a) and compared both the type specimens and the original descriptions and illustrations with the Mexican material that was labeled with these names. In this contribution we analyze and/or redescribe a group of South American species of *Eucyclops* including type specimens of *E. neumani*, *E. subciliatus*, and *E. pseudoensifer* deposited in the B.H. Dussart´s Collection in Paris, and *E. chilensis* and *E. breviramatus* from the Heinz Löffler's Collection in Wien. These species are taxonomically analyzed and compared with additional material and records found at F. Kiefer's Collection in Karlsruhe. We present an upgraded redescription of *Eucyclops pseudoensifer*, *E.*

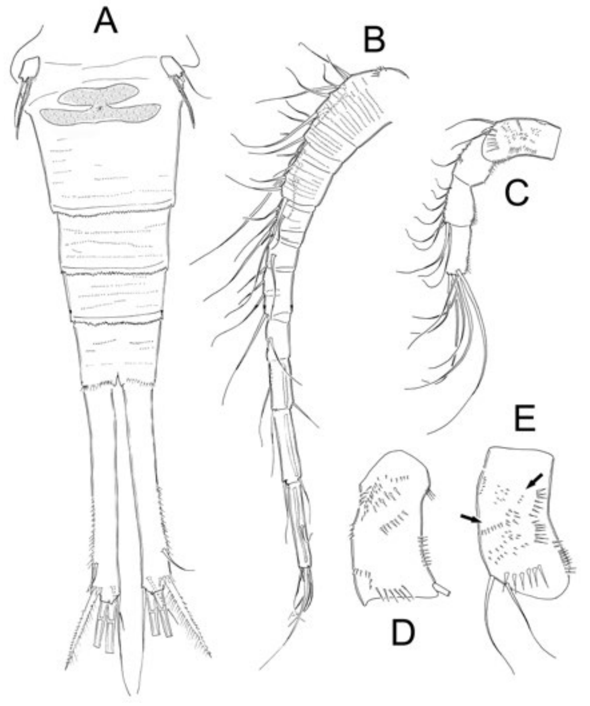

Figure 1. *Eucyclops neumani* s. str. (Pesta, 1927), adult female (MNHN-IU-2010-6807) from Argentina: A. urosome, ventral view; B. antennule; C. antenna; D. antennary basipodite, frontal view; E. antennary basipodite, caudal view. Eucyclops neumani *s. str. (Pesta, 1927), hembra adulta (MNHN-IU-2010-6807) de Argentina. A. urosoma, vista ventral; B. anténula; C. antena; D. basipodito de la antena, vista frontal; E. basipodito de la antena, vista caudal.*

neumani, *E. subciliatus*, and *E. breviramatus*. Due to editorial space constraints, the redescription of the taxonomically problematic *E. chilensis* will be provided in a further contribution.

MATERIALS AND METHODS

The redescription of both *E. pseudoensifer* and *E. subciliatus* was based on the examination of the type specimens from B. Dussart's collection deposited at the Muséum National d'Histoire Naturelle in Paris (France) (MNHN). Our redescription of *E. neumani* s. str. was based on the revision of samples from Argentina deposited in B.H. Dussart's collection; moreover, we compared our observations with F. Kiefer's accessory collection of drawings (Staatliches Museum für Naturkunde, Karlsruhe) of different populations of *E. neumani*.

The present redescription of *E. breviramatus* was based on the type specimens from H. Löffler's collection (Naturhistorisches Museum, Wien). Drawings were prepared at 1000× magnification with a Leitz Medilux compound microscope equipped with a drawing tube. Mapping and nomenclature of spinule and setule rows on the antennary basis, the coxopodite, and the intercoxal plate of P4 followed Alekseev *et al.* (2006) and Alekseev & Defaye's (2011) criteria. Abbreviations used in the descriptive section are: P1–P4 = first to fourth legs, Exp = exopod, Enp = endopod, s = seta/ae, ae = aesthetasc, sp = spine, Bsp = basipod, and CR = caudal ramus. The nomenclature used to describe the antennule and antennary armatures followed Alekseev *et al.* (2006), Alekseev & Defaye (2011), and Mercado-Salas *et al.* (2016). Caudal seta were labeled as follows: II—anterolateral (lateral) caudal seta, III—posterolateral (outermost) caudal seta, IV—outer terminal (terminal median external) caudal seta, V—inner terminal (terminal median internal) caudal seta, VI—terminal accessory (innermost) caudal seta, and VII—dorsal seta. The terms furca and telson were used following Schminke (1976). The museum specimens examined were either mounted on sealed slides or originally fixed and preserved in formalin, thus preventing the use of molecular analyses.

RESULTS

Order **Cyclopoida** (Rafinesque, 1815)
Family **Cyclopidae** (Rafinesque, 1815)
Subfamily **Eucyclopinae** (Kiefer, 1927)
Genus ***Eucyclops*** (Claus, 1893)
Eucyclops neumani **s. str.** (Pesta, 1927).
(Figures 1–4)

Material examined: adult female from Chaco, Argentina (MNHN-IU-2010-6807), St. R32, Arroyo del Tres; date of collection: September 19, 1984; collector S.M. Frutos; B. H. Dussart Collection, Muséum National d'Histoire Naturelle in Paris, France.

Female: average length excluding caudal setae = 1.34 mm. Urosome five-segmented (Fig. 1A), elongated; all urosomites ornamented with rows of

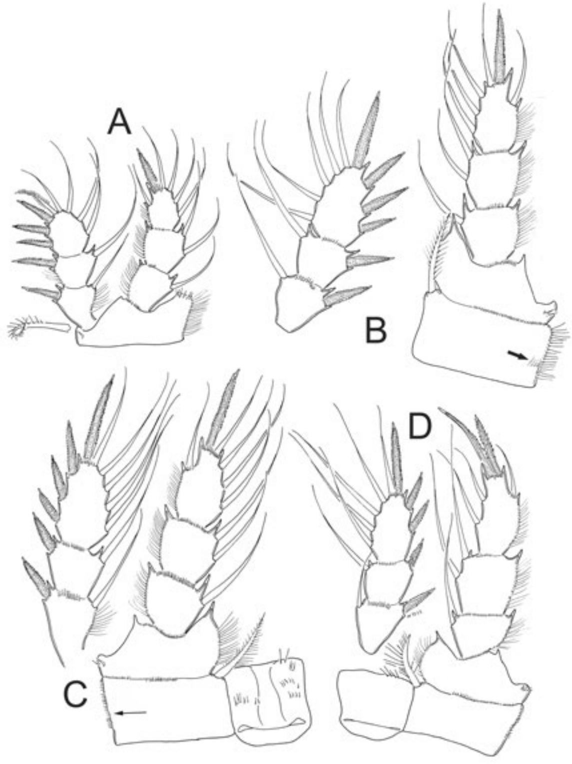

Figure 2. *Eucyclops neumani* s. str. (Pesta, 1927), adult female (MNHN-IU-2010-6807) from Argentina: A. leg 1; B. leg 2, exopodite separated for illustration; C. leg 3, idem; D. leg 4, idem. Eucyclops neumani *s. str. (Pesta, 1927), hembra adulta (MNHN--IU-2010-6807) de Argentina. A. pata 1; B. pata 2, el exopodito se ilustra por separado; C. pata 3, el exopodito se ilustra por separado; D. pata 4, el exopodito se ilustra por separado.*

cuticular pits. Posterior margin of telson with single row of spinules. Genital double-somite symmetrical. Seminal receptacle similar to the *serrulatus*-group, with narrow lateral arms on posterior margin. Genital somite 1.2 times longer than wide. Anal somite with cluster of small hair-like setules at each side of anal opening (see Fig. 33 in Dussart & Frutos, 1986), anal operculum rounded, with medial gap (see Fig. 33 in Dussart & Frutos, 1986; arrowed in Fig. 3B). Length/width ratio of CR = 8.4; inner margin of CR smooth. Serra covering 10–30 % of outer margin, with 6–15 spinules (Pesta, 1927; Dussart & Frutos, 1986; pers. obs. NM-S) (Figs. 1A, 3C–E). Dorsal seta (VII) short; about 0.25 times the length of CR and 0.60 times as long as outermost caudal seta (III). Length ratio of innermost caudal seta (VI)/outermost caudal seta (III) = 1.15. Lateral caudal seta (II) inserted at 80–88 % of CR. All terminal caudal setae plumose.

Antennule (A1) (Fig. 1B): 12-segmented. Armature per segment as follows: (s = seta, ae = aesthetasc, sp = spine): 1(8s), 2(4s), 3(2s), 4(6s), 5(3s), 6(1s + 1sp), 7(2s), 8(3s), 9(2s + 1ae), 10(2s), 11(3s), and 12(7s). Segments 1–8 furnished with rows of small pits (see Fig. 1B).

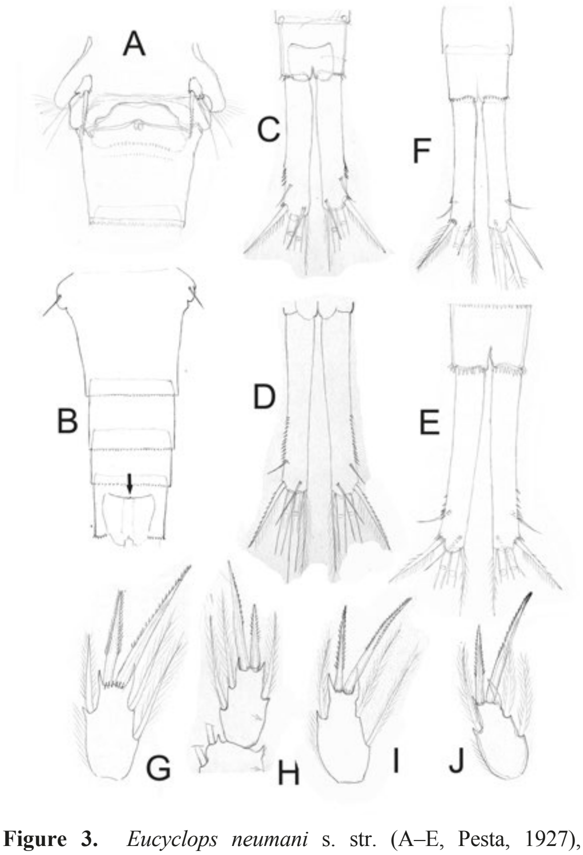

Figure 3. *Eucyclops neumani* s. str. (A–E, Pesta, 1927), *Eucyclops titicacae* (F–J, Kiefer, 1956): A. genital double somite (SMNK-11154); B. urosome, dorsal view (SNMK-02879); C. caudal rami (SNMK-11149); D. caudal rami (SNMK-02879); E. caudal rami (SNMK-11154); F. caudal rami (SNMK-05753); G. leg 4 (SNMK-03107); H. leg 4 (SNMK-11149); I. P4 (SNMK-02879); J. P4 (SNMK-02753). Eucyclops neumani *s. str. (Pesta, 1927) A-E, G-I;* Eucyclops titicacae *Kiefer, 1956 F, J. A. doble somita genital (SMNK-11154); B. urosoma, vista dorsal (SNMK-02879); C. ramas caudales (SNMK-11149); D. ramas caudales (SNMK- 02879); E. ramas caudales (SNMK-11154); F. ramas caudales (SNMK-05753); G. pata 4 (SNMK- 03107); H. pata 4 (SNMK-11149); I. pata 4 (SNMK-02879); J. pata 4 (SNMK-02753).*

Antenna (A2) (Figs. 1C–E): basis (2s + Exp), plus three-segmented Enp (armature: 1s, 9s, 7s). Ornamentations of basis (*sensu* Alekseev and Defaye, 2011) as follows: on frontal surface, N1(6), N2(6), N3(7), N4(20 in group), N5(6), N6(3), and N17(8); and on caudal surface N7(18 in group), N8(6), N9+10(9), N11(7), N12(6), N13(7 in group), N14(9 in group), N15(3), and N16(7) additional rows of spinules below N7 and another between N12 and N14 (both arrowed in Fig. 1E).

Mouthparts: not observable in slides.

P1–P4: Enp and Exp of all legs three-segmented. Armature formula as in Table 1.

P1 (Fig. 2A): intercoxal sclerite ornamentation not observable in slides. Basipodal spine reaching middle margin of Enp3 = 0.7 times as long as Enp. Length/width ratio of Enp3 P4 = 1.3; ratio of apical spine/length of Enp3 = 1.2.

P2 (Fig. 2B): intercoxal sclerite ornamentation not observable in slides. Coxa with row of hair-like setules along outer margin; one transversal row of spinules on middle margin, close to outer margin (caudal view) (arrowed in Fig. 2B), small spinules at insertion of Enp.

P3 (Fig. 2C): intercoxal sclerite with three rows of spinules on caudal surface: first row close to apical margin, bearing five spinules on each side; second row transverse, with tiny spinules along medial margin; third one a tramsverse a transversal row of small spinules located below the second row. Distal margin of intercoxal sclerite

Table 1. Setal formula of legs 1–4 in the type specimens of species of *Eucyclops* studied; spines in Roman numerals, setae in Arabic numerals; (–) represents structures not observed on the type material. *Fórmula setal de las patas 1-4 en los especímenes tipo de las especies de* Eucyclops *que fueron estudiadas; (espinas en números romanos, setas en números arábigos). (–) representa estructuras no observadas en el material tipo.*

Species		Coxa	Basis	Exp	Enp
***E. neumani* s. str.**	P1	0–1	1–I	I–1; I–1; III–5	0–1; 0–1;1–I–4
	P2	0–1	1–0	I–1; I–1; IV–5	0–1: 0–2: 1–I–4
	P3	0–1	1–0	I–1; I–1; IV–5	0–1; 0–2; 1–I–4
	P4	0–1	1–0	I–1; I–1; III–5	0–1; 0–2; 1–II–2
E. subciliatus	P1	0–1	1–I	I–1, I–1, III–5	0–1, 0–2, 1–I–4
	P2	0–1	1–0	I–1; I–1; IV–5	0–1; 0–2; 1–I–4
	P3	0–1	1–0	I–1, I–1, IV–5	0–1, 0–2, 1–I–4
	P4	0–1	1–0	I–1, I–1, III–5	0–1,0–2, 1–II–2
E. pseudoensifer	P1	0–1	1–I	I–1; I–1; III–3	0–1; 0–1;1–I–4
	P2	0–1	1–0	I–1; I–1; IV–5	0–1: 0–2: 1–I–4
	P3	0–1	1–0	I–1; I–1; IV–5	– ; – ; –
	P4	0–1	1–0	I–1; I–1; III–5	0–1; 0–2; 1–II–2

Table 2. Comparison of the surface ornamentation pattern of the antennary basis in some South American species of *Eucyclops*. Nomenclature of element groups follows Alekseev & Defaye (2011): Roman numerals = hairs, Arabic numerals = spinules, ? = structure not observed, NP = structure absent. *Comparación del patrón de ornamentación superficial de la base antenaria en algunas especies sudamericanas de* Eucyclops. *La nomenclatura de los grupos de elementos según Alekseev y Defaye (2011): números romanos = pelos; Números arábigos = espínulas; ? = estructura no observada; NP = estructura ausente.*

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
E. elegans (ECO-CH-Z-04948 Mexico)	XVI		9	8	5	NP	7	5	7		9	12	6	13	5	14	16
E. serrulatus “A” (Alekseev & Defaye, 2011)	VI–VIII	I–II	8–9	7–8	12–16	1–4	4–5	–	5–8	–	5–6	6–8	–	5–8	4–5	–	10–13
E. serrulatus “B” (Alekseev & Defaye, 2011)	V–IX	III–IV	7	9	13	4–5	3–4	–	5–6	–	5–6	7	2	3–4	4–5	–	9–10
E. speratus (Alekseev *et al*., 2006)	–	–	–	7	12	–	5–8	–	3	7	3–5	4–5	–	2–5	4–6	5	10–12
E. neumani s. str. (MNHN-IU-2010-6807)	6	6	7	20	6	3	18	6	9		7	6	7	9	3	7	8
E. titicacae (Alekseev & Defaye, 2011)	III	-	7	6	2	5	6	?	3	3	5	4	5	5	4	8	9
E. subciliatus	3	-	-	4	-	8	4	7	8		8	7	10	-	3	-	8
E. pseudoensifer	NP	NP	NP	5	?	?	5	4	8		5	9	4	?	4	4	8

with two slightly rounded projections. Coxa with strong biserially setulated coxal seta and tiny spinules along outer margin (arrowed in Fig. 2C). Unmodified seta on Enp3 and Exp3. All spines in Exp with tiny spinules at insertion.

P4 (Fig. 2D): intercoxal sclerite only, with row I observable, represented by four or five spinules on each side and a small medial gap. Inner coxal spine with heteronomous ornamentation: basally, with long hair-like setules; distally, with short spine-like setules; outer edge with hair-like setules on the proximal surface. Length/width ratio of Enp3 P4 = 2.2; length ratio of inner/outer spines of Enp3 P4 = 1.3; length ratio of inner spine of Enp3 P4/length Enp3 P4 = 1.1; length ratio of outer spine of Enp3 P4/length Enp3 P4 = 0.8. Lateral seta of Enp3 P4 inserted at 75 % of the total length of the segment. No modified setae on Enp3 and Exp3. Spines of Exp with tiny spinules at the insertion.

P5 (Fig. 1A): free segment subrectangular, 1.4 times longer than wide; bearing one inner spine and two setae; medial seta as long as outer seta; inner spine slightly longer than setae (about 1.1 times longer); inner spine 2.4 times longer than segment.

Remarks: The strikingly elongate CR is among the main characters to distinguish *Eucyclops neumani* s. str. from its congeners, but this feature is shared with other species like *E. elegans*, *E. speratus*, *E. macruroides* s. str. (Lilljeborg, 1901), *E. roseus* (Ishida, 1997), and *E. pacificus* (Ishida, 2000). Of these, only *E. elegans* is found in the Americas and can be easily distinguished from *E. neumani* s. str. because its serra covers more than 70 % of the CR outer margin, whereas in *E. neumani* s. str. this ornamentation covers only 30 % of the CR outer margin. Another character that clearly separates the two species is the shape and size of the P5 setal elements. In *E. elegans* the medial seta is always the longest and the outer one the shortest, the inner spine is long, strong, longer than the outer seta. A different pattern was found in *E. neumani* s. str. P5: the medial seta was as long as, or slightly longer than, the outer seta, and the outer seta is always as long as or slightly shorter than the inner spine; thus, in *E. neumani* s. str. the three elements of P5 are subequally long. The ornamentation of both the frontal and caudal surfaces of the antennary basis also differs between these two species. In *E. elegans* rows N1 and N2 bear long hairs and are not clearly separated, appearing as part of a single group; in *E. neumani* s. str. those two rows are clearly separated from each other and formed by small spinules. Both species present additional rows of spinules compared with the general pattern described by Alekseev *et al.* (2006), Alekseev & Defaye (2011), and Mercado-Salas *et al.* (2016). In *E. neumani* s. str. one extra row is found adjacent to group N7 and is probably part of group N22; another row was observed between groups N12 and N14, probably assignable to Mercado-Salas *et al.*'s (2016) group N20; in *E. elegans* only one extra row was found between groups N15 and N17 and is herein recognized as row N18.

During a visit to F. Kiefer's copepod collection (Staatliches Museum für Naturkunde, Karlsruhe), we examined his illustrations of the American *Eucyclops*. We found drawings of four populations of *E. neumani* s. str. and the original illustrations of *E. titicacae*. In Table 3, we provide a comparison of the main meristic characters resulting from the observation of this material. Also, we compared this data with the meristic values obtained from the examination of material from B. H. Dussart's collection and also with drawings by Pesta (1927) and Dussart & Frutos (1986). In this way, we were able to obtain a reasonably complete overview of the morphological variability of this species in its known distributional range and compare it with *E. neumani titicacae* in order to determine if the subspecific status of the latter is supported. In Kiefer's collection we examined illustrations of *E. neumani* s. str. from Uruguay (SMNK-03107), Lago Rupanco, Chile (SMNK-11149), Recife, Brazil (SMNK-02879), Río Cruce, Chile (SMNK-11154), and of *E. titicacae* from Lake Titicaca, Peru (SMNK- 05752, SMNK-05753, SMNK-05754). As shown in Table 3 and in Figures 3 and 4, most meristic values in this analysis have reduced variation ranges; only a few characters showed sizable variations among populations: (1) the CR length/width ratio range (5.8–8.4), (2) the P5 free segment length/width ratio range (1.0–2.0), and most importantly, (3)

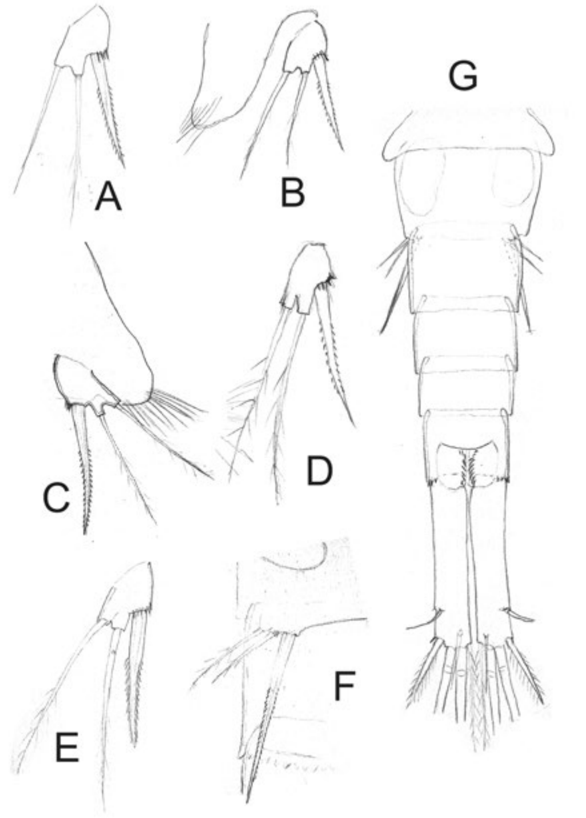

Figure 4. *Eucyclops neumani* s. str. (A–D, Pesta, 1927): *Eucyclops titicacae* (E–G, Kiefer, 1956): A. leg 5 (SNMK-03107), B. leg 5 (SNMK-11149); C. P5 (SNMK-02879); D. P5 (SNMK-11154); E. P5 (SNMK-05752); F. leg 6 (SNMK-05754); G. urosome, dorsal view (SNMK-05754). Eucyclops neumani *s. str. (Pesta, 1927) A-D;* Eucyclops titicacae *Kiefer, 1956 E-G. A. pata 5 (SNMK-03107), B. pata 5 (SNMK--11149); C. pata 5 (SNMK-02879); D. pata 5 (SNMK-11154); E. pata 5 (SNMK-05752); F. pata 6 (SNMK-05754); G. Urosoma, vista dorsal (SNMK-05754).*

the proportional length of the P5 medial seta/inner spine.

Based on these results, the latter character is arguably the main one to distinguish the strict form of *E. neumani* from its former subspecies *E. titicacae* (Figs. 4A–E). In *E. neumani* the medial spine is as long as or slightly shorter than the inner spine (0.8–1.0) and in *E. titicacae* the medial seta is always 1.3 times as long as or even longer than the inner spine. Because of this, it is likely that specimens from Uruguay (SMNK-03107-Fig. 4A) and Río Cruce, Chile (SMNK-11154-Fig. 4D) are assignable to *E. titicacae* and not to the strict form of *E. neumani*, although these records would be outside the presumed biogeographic range of *E. titicacae* (Mercado-Salas *et al.*, 2012; Fuentes-Reinés and Suárez-Morales, 2013). Fuentes-Reinés and Suárez-Morales (2013) proposed the scarce ornamentation of the CR outer margin found in *E. titicacae* to distinguish it from *E. neumani* s. str. In the former species there are always 3–5 spinules (Figs. 1A, 3C, D) whereas in *E. neumani* the number of spines covering the outer margin ranges between 7 and 15 (Fig. 3E, F) (see Fuentes-Reinés & Suárez-Morales, 2013, Fig. 4F). Kiefer's drawings of *E. titicacae* provided data about the ornamentation of the caudal surface of the P4 intercoxal sclerite, which differs from that of its known congeners but it was not possible to compare this pattern with the type specimens of *E. neumani* s. str. Records of *E. neumani* s. str. by Reid (1991) from Tres Lagunas, San Luis, Argentina were not included here because illustrations were not available to us.

Eucyclops subciliatus (Dussart, 1984).
(Figs. 5, 6)

Material examined: adult female from São Paulo, Brazil (NMHN-IU-2007-3270). Date of collection August 12, 1982; identified by B. H. Dussart; B. Dussart Collection, Muséum National d'Histoire Naturelle, Paris, France.

Female: habitus as in Figure 5A. Average length excluding caudal setae = 1.0 mm. Prosome expanded at first and second somites, representing 60 % of total body length, symmetrical in dorsal view. Pedigerous somites 2—4, with strongly developed subacute lateral pleural projections. Urosome five-segmented (Fig. 5B), relatively elongate; urosomal fringes lightly serrated; posterior margin of anal somite with row of strong spinules. Genital double-somite symmetrical, 1.2 times longer than wide. Seminal receptacle with narrow rounded lateral arms on posterior margin. Telson with short hair-like seta on anal opening, anal operculum serrated (Fig. 5C). Length/width ratio of CR = 4.7; hair-like setules present along the inner margin of CR, strong spinules covering

Table 3. Morphometric data of *E. neumani* s.str. obtained from: (1) Pesta (1927), (2) Dussart & Frutos (1986), (3) Uruguay (SMNK-03107), (4) Lago Rupanco, Chile (SMNK-11149), (5) Recife, Brazil (SMNK-02879), (6) Río Cruce, Chile (SMNK-11154), (7) Dussart (MNHN-IU-2010-6807), and (8) *E. titicacae* from Lake Titicaca, Peru (SMNK-05752, SMNK-05753, and SMNK-05754). *Datos morfométricos de* E. neumani *s.str. obtenido de: 1) Pesta (1927), 2) Dussart & Frutos (1986), 3) Uruguay (SMNK-03107), 4) Lago Rupanco, Chile (SMNK-11149), 5) Recife, Brasil (SMNK-02879) , 6) Río Cruce, Chile (SMNK-11154), 7) Dussart (MNHN-IU--2010-6807) y 8)* E. titicacae *del lago Titicaca, Perú (SMNK-05752, SMNK-05753, SMNK-05754).*

Character	1	2	3	4	5	6	7	8
Length/width CR	8.0	7.5	-	5.8	7.6	7.7	8.4	6.5
VII/length CR	-	0.3	-	0.3	0.30	0.3	0.3	0.3
VII/III setae CR	-	0.7	-	0.6	0.7	0.8	0.6	0.7
VI/IV setae CR	1.3	1.2	-	1.0	1.1	1.0	1.15	1.0
Length/width Enp3 P4	-	2	2	2	2.1	-	2.2	1.9
Inner spine/length Enp3 P4	-	1.2	1.5	1.4	1.3	-	1.1	1.5
Outer spine/length Enp3 P4	-	0.9	1.0	0.9	0.9	-	0.8	1.0
Inner/outer spines Enp3 P4	-	1.3	1.5	1.6	1.3	-	1.3	1.4
Length/width P5	1.0	1.3	1.3	1.3	1.4	2.0	1.4	1.5
Medial/outer setae P5	1.2	1.0	1.1	0.8	1.0	1.0	1.0	1.0
Medial seta/inner spine P5	1.0	0.9	1.3	1.0	1.0	1.3	0.8	1.3
Inner spine/length P5	2.5	2.4	2	1.9	2.1	1.6	2.4	2.1

62 % of outer margin of CR. Dorsal seta (VII) short: 0.6 times the length of CR, and 0.7 times as long as outermost caudal seta (III). Ratio of innermost caudal seta (VI)/ outermost caudal seta (III) = 1.1. Lateral caudal seta (II) inserted at 82 % of CR length. All terminal setae plumose.

Antennule (A1) (Fig. 5D): 12-segmented, reaching middle margin of second prosomite; three distal segments with narrow hyaline membrane. Armament per segment as follows: 1(8s), 2(4s), 3(2s), 4(6s), 5(1s), 6(1s + 1sp), 7(2s), 8(3s), 9(1s), 10(2s), 11(2s), 12(7s). Two rows of spines on first segment, first row with small spinules and second row with stronger and longer spines.

Antenna (A2) (Fig. 5E, F): basis (2s + Exp), plus three-segmented Enp (armature: 1s, 8s, 7s). Basis ornamentation (sensu Alekseev and Defaye, 2011) as follows: on frontal surface: N1(3), N4(4), N6(8), N17(8); on caudal surface, N7(4), N8(7), N9 + 10(8), N11(8), N12(7), N13(10), N15(3).

Mouthparts: not observable in slides examined.

P1–P4: endopod and exopods of all swimming legs three-segmented. Armature formula as in Table 1.

P1 (Fig. 6A): intercoxal sclerite with cluster of tiny spinules close to apical margin on each side of frontal surface; distal margin with two rounded

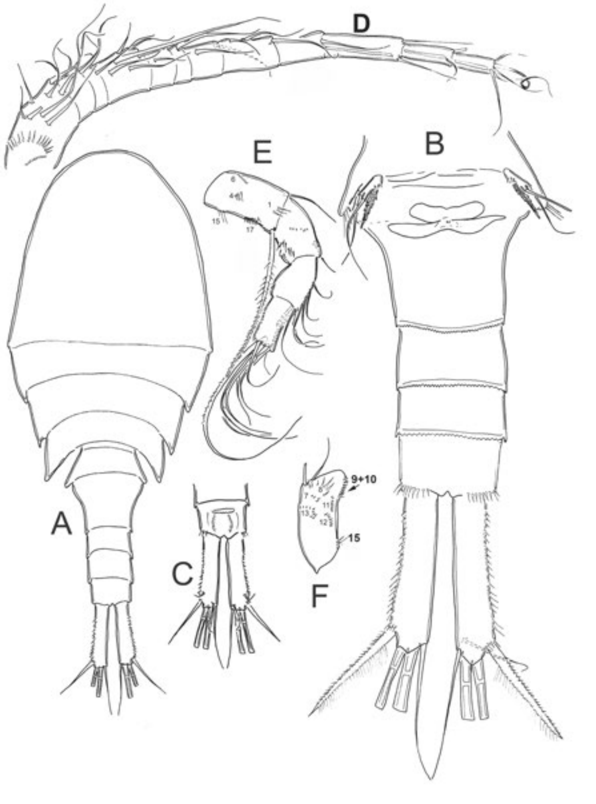

Figure 5. *Eucyclops subciliatus* (Dussart, 1984), adult female (MNHN-IU-2007-3270) from Sâo Carlos, Sâo Paulo, Brazil: A. habitus, dorsal; B. urosome, ventral; C. anal somite, dorsal; D. antennule; E. antenna, frontal view; F. antenna basipodite, caudal view. Eucyclops subciliatus *Dussart, 1984, hembra adulta (MNHN-IU-2007-3270) de Sâo Carlos, Sâo Paulo, Brazil. A. hábito, vista dorsal; B. urosoma, vista ventral; C. somita anal, vista dorsal; D. anténula; E. antena, vista frontal; F. basipodito de la antena, vista caudal.*

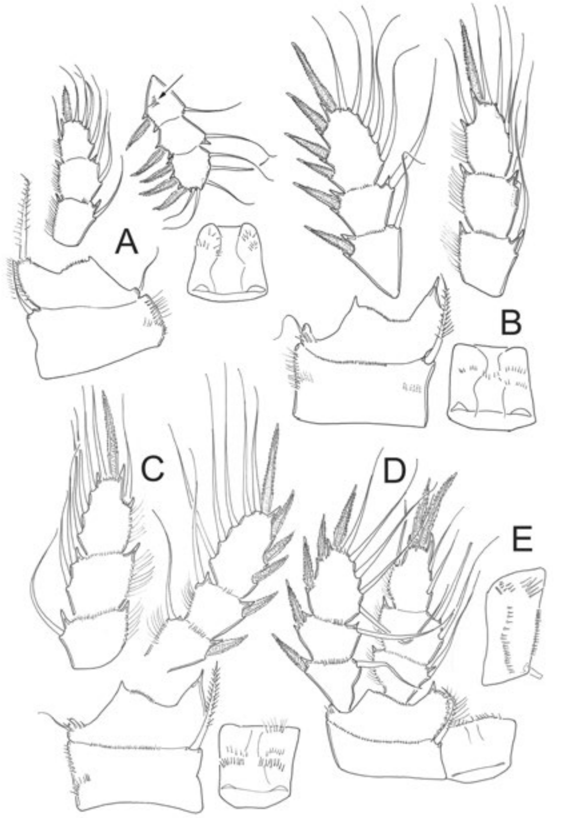

Figure 6. *Eucyclops subciliatus* (Dussart, 1984), adult female (MNHN-IU-2007-3270) from Sâo Carlos, Sâo Paolo, Brazil: A. leg 1; B. leg 2, rami separately illustrated; C. leg 3idem; D. leg 4; E. coxa, caudal view. Eucyclops subciliatus *Dussart, 1984, hembra adulta (MNHN-IU-2007-3270) de Sâo Carlos, Sâo Paulo, Brazil. A. pata 1; B. pata 2, ramas ilustradas por separado; C. patas 3, ramas ilustradas por separado; D. pata 4; E. coxa de pata 4, vista caudal.*

chitinized projections. Coxa with strong, biserially setulated inner coxal seta. Coxa with row of long setules along outer margin and transverse row of few spinules on middle margin, close to outer margin. Basipodal spine stout, reaching middle margin of Enp3, 0.7 times as long as Enp. Length/width ratio Enp3P4 = 1.2; proportion of apical spine/length of Enp3 = 0.9. Exp1 with row of small spinules on middle margin (arrowed in Fig. 6A).

P2 (Fig. 6B): intercoxal sclerite with two rows of tiny spinules along middle margin of caudal surface. Distal margin of intercoxal sclerite with two low, rounded, chitinized projections. Coxa with strong, biserially setulated inner coxal seta. Coxa with row of hair-like setules along outer margin. Small spinules at insertion of basipod on frontal surface. Caudal surface with two transverse rows of small spinules on middle margin: the first close to outer margin and the other close to inner margin. Small spinules on Exp at insertion of spines, with regular setae on Exp and Enp.

P3 (Fig. 6C): caudal surface of intercoxal sclerite with three rows of hair/spinules, all rows with small gaps at the center: the first close to the apical margin, bearing 14 hair-like setules; the second, a transverse row of 14 tiny spinules along medial margin; and the third one a transverse row of 18 spinules located next to second row. Distal margin of intercoxal sclerite with two slightly rounded projections. Coxa with strong biserially setulated coxal seta, coxa with spinules along outer margin, tiny spinules at insertion of basipod. Unmodified setae on Exp and Enp, with Enp segments slightly expanded.

P4 (Fig. 6D, E): intercoxal sclerite with row I observable, represented by seven small spinules on each side, with a small gap between them. Inner coxal spine with heteronomous ornamentation: basally, with long hair-like setules, and distally, with spine-like setules; outer edge with distal spine-like setules. Caudal coxal surface with spinule formula A + H, B, C + D, E, G. Length/width ratio of Enp3 P4 = 1.5; length ratio of inner/outer spines of Enp3 P4 = 1.2; length ratio of inner spines of Enp3 P4/length Enp3 P4 = 1.4; length ratio of outer spine of Enp3 P4/length Enp3 P4 = 1.1. Lateral seta of Enp3 P4 inserted at 70 % of segment length. Unmodified setae on Enp and Exp. Enp1 and 2 expanded (wider than long).

P5 (Fig. 5B): free segment subrectangular, twice as long as wide; bearing strong inner spine and two setae; medial seta 1.2 times longer than outer seta and 2 times longer than inner spine. Inner spine 1.6 times as long as segment.

Remarks: This species is easily recognizable from its congeners by the presence of setules along the inner margin of the furca; this charac-

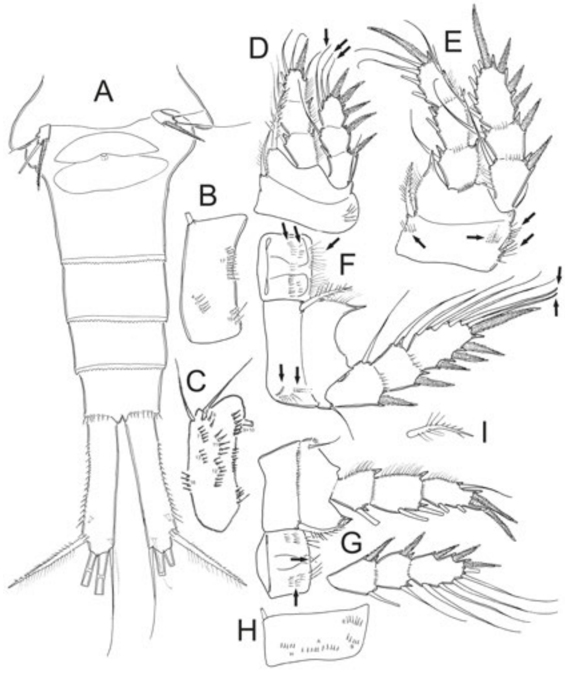

Figure 7. *Eucyclops pseudoensifer* (Dussart, 1984), holotype adult female (MNHN-IU-2007-3117) from Laguna Macabaji, Venezuela: A. urosome, ventral; B. antennary basipodite, frontal view; C. antennary basipodite, caudal view; D. leg 1; E. leg 2; F. leg 3; G. leg 4; H. Coxa, caudal view; I. inner coxal spine. Eucyclops pseudoensifer *Dussart, 1984, hembra adulta, holotipo (MNHN-IU-2007-3117) de Laguna Macabaji, Venezuela. A. urosoma, vista ventral; B. basipodito de la antena, vista frontal; C. basipodito de la antena, vista caudal; D. pata 1; E. pata 2; F. pata 3; G. pata 4; H. coxa, vista caudal; I. espina coxal interna.*

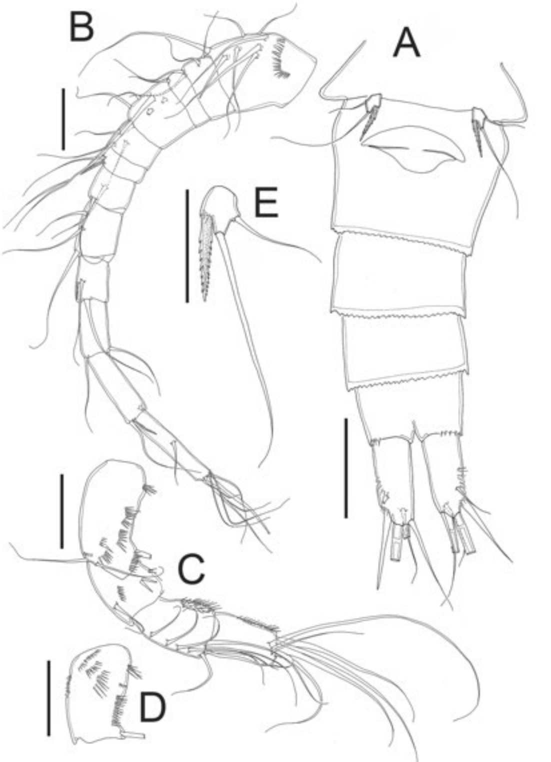

Figure 8. *Eucyclops pseudoensifer* (Dussart, 1984), allotype adult male (MNHN-IU-2007-3117) from Laguna Macabaji, Venezuela: A. antenna, frontal view; B. legs 5 and 6, ventral view; C. P1; D. P2; E. P4. Eucyclops pseudoensifer *Dussart, 1984, macho adulto, alotipo (MNHN-IU-2007-3117) de Laguna Macabaji, Venezuela. A. antena, vista frontal; B. patas 5 y 6, vista ventral; C. pata1; D. pata 2; E. pata 4.*

ter is not found in any other American species of *Eucyclops*, and it is shared with the African *E. ciliatus* only. The ornamentation pattern of the frontal and caudal surfaces of the antennary basis was included in Table 2 in order to compare it with its closest congeners.

Eucyclops pseudoensifer (Dussart, 1984)
(Figs. 7-8)

Material examined: holotype adult female from Laguna Macabaji, Venezuela; specimen dissected (NMNH-IU-2007-3117), September 5, 1980, by Evelyn Zoppi. Allotype adult male specimen dissected (NMNH-IU-2007-3116), same collection date and collector. B.H. Dussart Collection (Muséum National d'Histoire Naturelle, Paris, France).

Female: Average body length excluding caudal setae = 0.8 mm. Prosome expanded at first and second somites, representing 58 % of total body length, symmetrical in dorsal view. Five segmented urosome, relatively elongated; posterior margin of telson with one row of strong spinules. Genital somite symmetrical, 1.1 times longer than wide. Seminal receptacle as in serrulatus-group (Fig. 7A). Anal operculum slightly rounded. Length/width ratio of CR = 4.3; spinules along outer margin, increasing in size as they approach the apical margin, covering 63–65 % of length of furca. Dorsal seta (VII) short: 0.3–0.4 times the length of CR and, 0.4–0.6 times as long as outermost caudal seta (III). Ratio of innermost caudal seta (VI)/outermost caudal seta (III) = 1.1–1.2. Lateral seta (II) inserted at 78 % of furca. All terminal setae plumose.

Antennule (A1): 12- segmented, not reaching posterior margin of cephalothorax; last three segments with narrow hyaline membrane. Armament per segment as follows: 1(8s), 2(4s), 3(2s), 4(6s), 5(3s) 6(1s + 1sp), 7(2s), 8(3s), 9(2s + 1ae), 10(2s), 11(2s), 12(7s).
A2 (Figs. 7B, C): Basis (2s + Exp), three-segmented Enp (armature: 1s, 9s, 7s). Basis ornamentations sensu Alekseev and Defaye (2011) as follows: on frontal surface, N4(5) N15(4); on caudal surface, N7(5), N8(4), N9 + 10(8), N11(5), N12(9), N13(4), N15(4), N16(4), N17(8) (see Suárez-Morales & Walsh, 2009, Fig. 6A).

Mouthparts: not observable in slides.

P1–P4: endopod and exopods of all swimming legs three-segmented. Armature formula as in Table 1.
P1 holotype (Fig. 7D): coxa with strong biserially-setulated inner coxal seta. Basipodal spine not reaching medial margin of Enp3, 0.6 times as long as Enp. Length/width ratio of Enp4 = 1.4; apical spine as long as segment (Enp3). Holotype with abnormal setation pattern on Exp3, as outlined by Dussart (1984), with only three inner setae (arrow in Figure 7D). Paratype (Suárez-Morales & Walsh, 2009, Fig. 6B): intercoxal sclerite with a group of strong and relatively long spinules on each side of frontal surface and distal margin with two rounded chitinized projections. Coxa with strong and biserially-setulated inner coxal seta. Basipodal spine not reaching medial margin of Enp3, 0.8 times as long as Enp. Length/width ratio Enp3 = 1.4; apical spine as long as segment (Enp3). Paratype with the normal pattern of setation on Exp3, bearing five setae on inner margin.
P2 holotype (Fig. 7E): coxa with strong biserially setulated inner coxa seta. Coxa with two rows of long hair spinules along outer margin, one below the other (arrowed in Fig. 7E). Caudal surface of coxa with two transversal rows of long spinules in middle margin: one close to outer margin and the second close to inner margin (arrowed in Fig. 7E). Length/width ratio of Enp3 = 1.6: apical spine 1.5 times as long as segment (Enp3). Unmodified setae on Enp and Exp. Paratype (Suárez-Morales & Walsh, 2009, Fig. 6B): frontal surface of intercoxal sclerite with a group of hairs close to apical margin on each side of sclerite. Coxa with strong biserially setulated inner coxa seta. Coxa with one row of spinules along outer margin. Length/width ratio of Enp3 = 1.7; apical spine 1.4 times as long as segment (Enp3). Unmodified setae on Enp and Exp.
P3 holotype (Fig. 7F): caudal surface of intercoxal sclerite ornamented with three spinule rows: the first bearing long hairs with small medial gap; second row with short, strong spinules; third one furnished with strong, short spinules (all arrowed

in Fig. 7F). Caudal surface of coxa with two rows of spinules close to outer margin: the first bearing long spinules and the second (below the first) armed with tiny spinules (both arrowed in Fig. 7F). Length/width ratio of Enp3 = 2.0, apical spine 1.2 times as long as segment (Enp3). Two most apical setae of Exp modified (arrowed in Fig. 7F).

Paratype (Suárez-Morales & Walsh 2009, Fig. 6D): frontal surface of intercoxal sclerite with a group of hair-like setules close to apical margin on each side of sclerite. Coxa with strong biserially setulated inner coxa seta. Coxa with one row of small, strong spinules along outer margin. Length/width ratio of Enp3 = 2.0, apical spine 1.3 times as long as segment (Enp3). Unmodified setae on Enp and Exp.

P4 holotype (Figs. 7G–I): intercoxal sclerite with only row I observable on caudal surface, represented by five slender, elongated spinules on each side, separated by a gap (arrowed in Fig. 7G); frontal surface with a group of 19 hairs arranged in a circle close to apical margin on each side of sclerite (arrowed in Fig. 7G). Inner coxal spine with heterogeneous ornamentation: basally, with long hair-setules, and distally, with spinules; outer edge with four hair setules set basally and distally with one strong spinule and medial gap (arrowed in Fig. 7I). Frontal surface of coxa with row of tiny spinules at insertion of basipod, caudal surface of coxa with spinule formula: A+B+E+H (rows C+D—G, J not observed). Length/width ratio of Enp3 = 2.0; length ratio of inner spine/outer spine of Enp3 = 1.2; length ratio of inner spine/length of Enp3 = 1.2, length ratio of outer spine/length of Enp3 = 1.0. Lateral seta of Enp3 inserted at 72 % of the total length of segment. Unmodified seta on Enp and Exp. Paratype (Suárez-Morales & Walsh, 2009, Fig. 7A, C): intercoxal sclerite with only row I drawn, represented by seven long hairs on each side, separated by gap. Inner coxal seta with uniform ornamentation. Length/width ratio of Enp3 = 2.3; length ratio of inner/outer spines of Enp3 = 1.2; length ratio of inner spine/length of Enp3 = 1.2, length ratio of outer spine/length of Enp3 = 1.0. Lateral seta of Enp3 inserted at 71 % of the total length of segment. Unmodified setae on Enp and Exp.

P5 holotype (Fig. 7A): free segment subrectangular, 1.7 times as long as wide; bearing one inner spine and two setae; medial seta 2.1 times longer than outer seta and 1.3 times longer than inner spine. Inner spine 2.6 times longer than segment. Paratype (Suárez-Morales & Walsh, 2009 Fig. 5B): free segment subrectangular, 1.6 times longer than wide; bearing one strong inner spine and two setae; medial seta 2.3 times longer than outer seta and 1.3 times longer than inner spine. Inner spine 2.7 times longer than segment.

Remarks: This species was recently redescribed by Suárez-Morales and Walsh (2009) using type material (paratypes). Most of the characters used in the current taxonomy of *Eucyclops* were considered in the redescription, except for the ornamentations of the intercoxal sclerites (caudal surface) of the third and fourth legs and the ornamentation of the fourth coxa and inner coxal seta; these details are first described herein. We found some differences between the paratypes and the holotype specimen. The holotype shows the anomalous setation of leg 1 described by Dussart (1984a), in which Exp3 bears only 3 setae instead of the normal pattern of 5 setae (Suárez-Morales & Walsh, 2009) in the paratype. The intercoxal sclerite of the fourth swimming leg has an additional difference: in the holotype specimen, row I has long spinules whereas in the paratype this row bears long and slender hair-like elements. In the Mexican material examined we found a particular pattern in the intercoxal sclerites of legs 3 and 4, with row I of P4 bearing hair-like elements; also, P3 caudal rows I-III are always represented by hair-like elements as well, but when Row I of P4 bears spinules, at least one of the three rows of P3 has spinules. If we assume that this pattern is general among the American *Eucyclops*, the holotype and paratypes of *E. pseudoensifer* represent an exception to this rule. In the holotype the intercoxal sclerite follows the pattern described, with three rows on the caudal surface, one row with fine and long hair-like elements and the other two with strong but small spinules. The ornamentation of the intercoxal sclerites has been used in several genera of cyclopid copepods (Cyclops, Acanthocyclops, Mesocyclops, Microcyclops) for the separation of closely related species (Einsle, 1985; Rocha, 1998), but most attention has

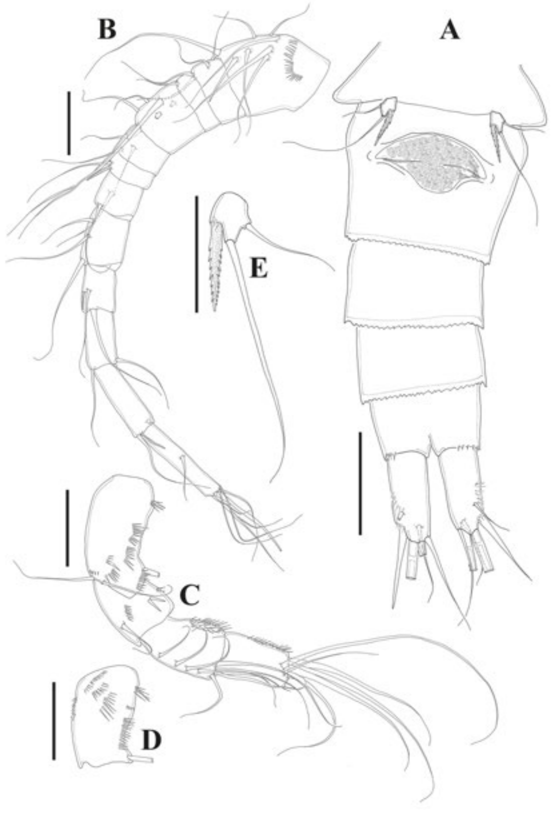

Figure 9. *Eucyclops breviramatus* (Löffler, 1963), syntypic female specimen from Ecuador: A. urosome, ventral view; B. antennule, frontal view; C. antenna; D. antennary basis, caudal view; E. P5, ventral view. Eucyclops breviramatus *Löffler, 1963, especimen sintípico, hembra de Ecuador. A. urosoma, vista ventral; B. anténula, vista frontal; C. antena; D. basipodito de la antena, vista caudal; E. pata 5, vista ventral.*

been given to the fourth leg. The evaluation of the sclerite ornamentation as a taxonomically valuable character to distinguish species of *Eucyclops* should be expanded to include all swimming legs.

Our results indicate that *E. pseudoensifer* does not belong to the serrulatus-group because it lacks rows N1 and N2 on the frontal surface of the antennal basis, a character pointed out by Alekseev & Defaye (2011) as distinctive of the serrulatus-group. Thus, this species can be easily distinguished from its American congeners with a similar CR: *E. bondi*, *E. pectinifer*, *E. prionophorus*, *E. cuatrocienegas* and *E. chihuahuensis*) by characters of the antennary basis. Another American species that is not assignable to the serrulatus-group is *E. conrowae*, which clearly differs from *E. pseudoensifer* by its remarkably long dorsal caudal seta (as long as or slightly shorter than the furca) while in *E. pseudoensifer* the dorsal seta is just 0.3-0.4 as long as the furca. In addition, the presence of strongly modified setae on the Enp and Exp of P4 is a distinctive character present in *E. conrowae vs.* normal setae in *E. pseudoensifer*.

Eucyclops breviramatus Löffler, 1963
(Figs. 9, 10)

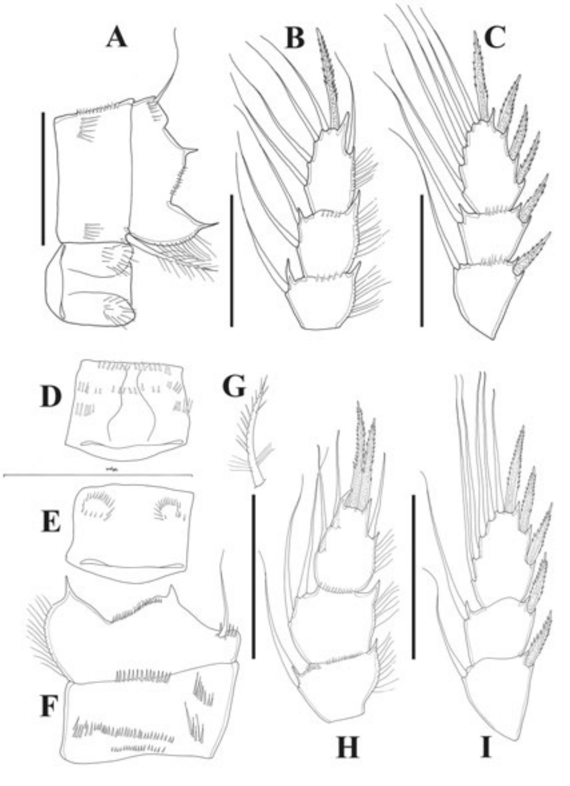

Figure 10. *Eucyclops breviramatus* (Löffler, 1963), syntypic female specimen from Ecuador: A. coxa and basis of P3, B. enp of the same, C. exp of the same; D. intercoxal sclerite of the same, caudal view; E. P4 intercoxal sclerite, caudal view; F. coxa and basis of the same; G. inner coxal spine of the same; H. enp of the same; I. exp of the same. Eucyclops breviramatus *Löffler, 1963, especimen sintípico hembra, de Ecuador. A. coxa y base de pata P3, B. endopodito de pata 3, C. exopodito de pata 3; D: esclerito intercoxal de pata 3, vista caudal; E. esclerito intercoxal de pata 4, vista caudal; F. coxa y base de pata 4; G espina coxal interna de pata 4; H. endopodito de pata 4; I. exopodito de pata 4.*

Material examined: syntypic specimens from H. Löffler's collection NHMW22245 (♀), NHMW22247 (♀), NHMW22250 (1♀, 1♂), collected by Herrn Prescott from Papallacta, Napo Province, Ecuador. Date: May 1st, 1958. No slide or specimen identified as the holotype; slide NHMW22250 includes 2♀♀ of Metacyclops mendocinus.

Female: Body length excluding caudal setae = 1164 µm (measurement taken from Löffler, 1963). Proportions between prosome and urosome not available (all specimens were dissected). 5-segmented ursome, not elongated (Fig. 9A); telson posterior margin with row of spinules. Genital double-somite symmetrical, as long as wide. Seminal receptacle not clearly defined. Anal operculum smooth, rounded. Length/width ratio of CR ranging from 2.37 to 2.61 (x = 2.46 m, n = 6); spinules on outer margin about the same size, covering 40 % of the total length of the furca. Dorsal seta (VII) about 0.8 times as long as CR and 0.8–0.9 times as long as outermost caudal seta (III). Ratio of innermost caudal seta (VI)/outermost caudal seta (III) = 1.0-1.1. Lateral seta (II) inserted at 62 % of CR length.

Antennule (A1) (Fig. 9B): 12-segmented, distal three segments with narrow hyaline membrane. Armament per segment as follows: 1(8s), 2(4s), 3(1s), 4(6s), 5(3s), 6(1s + 1sp), 7(2s), 8(3s), 9(2s +1ae), 10(2s), 11(2s + 1ae), 12(8s).
Antenna (A2) (Fig. 9C, D): Basis (2s+Exp), 3-segmented Enp (1s, 8s, 6s). Basis ornamentations confirmed in slides NHMW22247 and NHMW22250: frontal surface N3(5-8), N4(6-7), N5(12-15), N6(4), N15(5-7); on caudal surface, N7(5), N8(6–7), N9 + N10(9), N11(6-–7), N12(9), N14(4), N18(5-–6).

Mouthparts, **P1** and **P2** not observable in examined slides.

P3 (Fig. 10A–C): caudal surface of coxa, basis, and intercoxal sclerite not observable. Frontal surface of coxa ornamented with rows A, B, and C; frontal surface of intercoxal sclerite with fine setules arranged in circular pattern on each side. Coxa with strong, biserially setulated inner coxal seta. Length/width radio of Enp3 = 1.8; apical spine 1.2 times as long as segment (Enp3). Unmodified setae on Exp and Enp.
P4 (Fig. 10D–I): caudal surface of coxa with rows A, B, C + D, E, and H. Frontal surface of intercoxal sclerite with small spinules arranged in circular pattern on each side. Caudal surface of intercoxal surface with rows I, II, and III bearing spinules: row I continuous, bearing 15 spinules; row II with two small gaps dividing the row into three groups; row III with six spinules on each

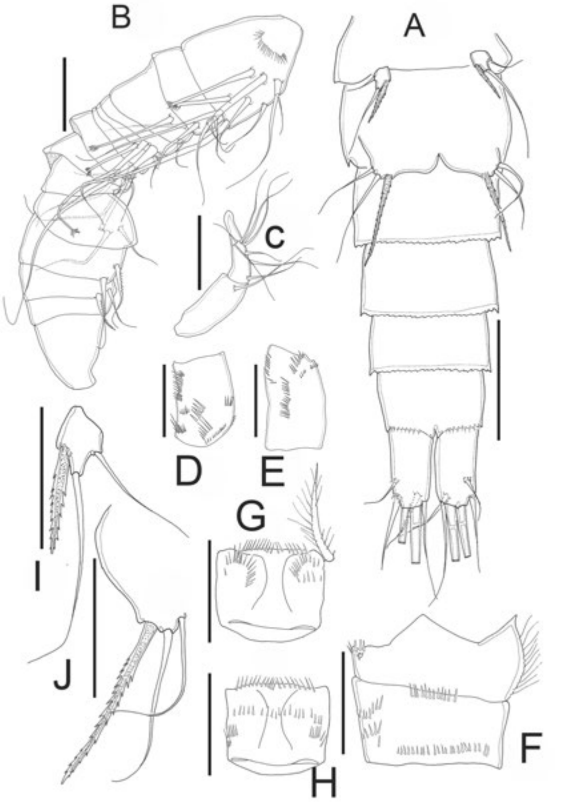

Figure 11. *Eucyclops breviramatus* (Löffler, 1963), syntypic male specimen from Ecuador: A. urosome, ventral view; B. antennule; C. distal segments of antennule; D. antennary basis, frontal view; E. the same, caudal view; F. P4 coxa and basis; G. P4 intercoxal sclerite, caudal; H. coxa and basis of same; I. leg P5, ventral view; J. leg 6, ventral view. Eucyclops breviramatus *Löffler, 1963, especimen macho sintípico de Ecuador. A. urosoma, vista ventral; B. anténula; C. segmentos distales de la anténula; D. base de la antena, vista frontal; E: base de la antena, vista caudal; F. coxa y base de pata 4; G esclerito intercoxal de la pata 4, vista caudal; H. coxa y basis de la pata 4; I. pata P5, vista ventral; J. pata 6, vista ventral.*

side, not continuous. Intercoxal spine with heterogeneous ornamentation: basally, with long hair-like setules, and distally, with spinules; outer edge with four hair-like setules set basally and distally, with two spinules, gap in the middle margin. Length/width ratio Enp3 = 1.5; length inner spine/outer spine Enp3 = 1.2; length inner spine/length of Enp3 = 1.25; length outer spine/length of Enp3 = 1.1. Lateral seta of Enp3 inserted at 68 % of the total length of segment. Three most apical setae of Exp3 modified.

P5 (Fig. 9E): one subrectangular free segment, 1.3 times as long as wide, bearing one inner spine and two setae; medial seta 2.1 times longer than outer seta and 2.7 times longer than inner spine. Inner spine 1.9 times longer than segment.

Male: body length excluding caudal seta 980 µm (from Löffler, 1963). Proportions between prosome and urosome not available; urosome 6-segmented (Fig. 11A). CR 2-2.4 times longer than wide; medial margin naked, spinules at insertion of caudal seta (III) and outermost terminal causal seta (III). Dorsal seta (VII) as long as CR and, slightly longer than outermost caudal seta (III). Lateral caudal seta (II) inserted at 75 % of caudal ramus.

Antennule (Fig. 11B, C): 16-segmented, armature per segment as follows: 1(7s + 2ms), 2(4s), 3(1s + 1ms), 4(1s +1ms), 5(1s + 1ms), 6(1s), 7(2s), 8(1S), 9(2s), 10(2s), 11(2s), 12(2s), 13(0), 14(0), 15(1s), 16(8s).

Antenna (Fig. 11D, E): basis with spinule groups: on frontal surface, N3(5), N4(6),N5(11), N6 (4), 15(7), 17(14), 18(6), and on caudal surface, N7(7), N8(9), N9 + N10(9), N11(6), N12(9).

P4 (Fig. 11F–H): as in female.

P5 (Fig. 11I): Free segment subrectangular, 2.5 times longer than wide, bearing one spine and two setae; medial seta longer than outer seta (about 1.6 times) and inner spine (1.9 times). Inner spine slightly shorter than outer seta.

P6 (Fig. 11J): represented by a small, low plate adjacent to lateral margin of genital somite, armed with one strong inner spine and two unequally-sized setae. Inner seta not reaching medial margin of fourth urosomite; inner spine 1.1 times longer than medial seta and 1.3 times longer than outer seta.

Remarks. *Eucyclops breviramatus* was described by Löffler (1963) from material collected in Ecuador; was subsequently reported in Mexico, but Mexican records of this species are probably assignable to one of the species recently described by Mercado-Salas *et al.*, 2016).

Our observations indicate that *E. breviramatus* should not be considered a member of the *E. serrulatus*-complex as it lacks rows N1 and N2 on the antennary basis, which is a distinctive character present only in species of this group (Alekseev *et al.*, 2006; Alekseev & Defaye, 2012).

DISCUSSION

In 1927 Otto Pesta described *Eucyclops neumani* from Argentina; this species closely resembles the cosmopolitan *E. macrurus* (Sars, 1863) and *E. serrulatus*, but clearly differs from both. After its original description, *E. neumani* has been recorded in Argentina, Brazil, Chile, Peru, and Uruguay (Reid, 1985, 1991; Dussart & Frutos, 1986; Rocha & Botelho, 1998; Dussart & Defaye, 2006). Friedrich Kiefer (1957) described the subspecies *Eucyclops neumani titicacae* from Lake Titicaca, Peru; it has been recorded thereafter in Venezuela (Lake Valencia), Bolivia and Peru (Lake Titicaca) (Reid, 1985; Del Río & Valdivia, 1989; Silva, 2008), and recently from Colombia, an illustrated record in which *E. titicacae* is recognized as an independent species (Fuentes-Reinés & Suárez-Morales, 2013).

One of the main researchers of the freshwater copepod fauna worldwide, particularly of the tropical regions, was the French copepodologist Bernard H. Dussart. His works revealed much of the rich tropical biodiversity and created a new stimulus for the study of freshwater copepods (Defaye & Vaupel Klein, 2011). During the course of his long career, he described many freshwater cyclopoid species of *Eucyclops* from South America; *Eucyclops pseudoensifer* Dussart, 1984 and *E. subciliatus* Dussart, 1984 are among them. The former was collected from the Venezuelan Andes. This taxon resembles the Chilean *E. ensifer* (Kiefer, 1936), but differs in the structure of the female fifth leg and male sixth leg. After its original description, *E. pseudoensifer* was recorded in Mexico, Colombia, and Venezuela (Reid, 1985; Gaviria, 1994; Grimaldo-Ortega *et al.*, 1998; Mercado-Salas, 2009). In the same year, Dussart (1984a) described another species of *Eucyclops* from Brazil: *E. subciliatus*, collected from a pond near Sâo Carlos, which closely resembled the African *E. ciliatus* (G.O. Sars, 1909) for its sharing of caudal rami furnished with hair-like setules on the inner margins—a rare character in the genus—and *E. serrulatus* as described by Smith and Fernando (1980) from Cuba. This species was recorded only from Brazil and Argentina (Dussart & Frutos, 1986; Rocha & Botelho, 1998), but local and regional records of *E. serrulatus* should be revised in the light of the present redescription. *Eucyclops pseudoensifer* was redescribed by Suárez-Morales and Walsh (2009) using current upgraded standards and an examination of the paratypes; it was found to resemble both the South American *E. silvestrii* (Brian, 1927) and the North American *E. chihuahuensis* (a presumed Mexican endemic; Suárez-Morales & Walsh, 2009), but had greater affinity with to the latter. Because of the lack of rows N1and N2 on the antennary basis, this species is not assignable to the *E. serrulatus* species-group (Mercado-Salas *et al.*, 2016).

The available descriptions of both *E. neumani neumani* and *E. subciliatus* are in need to be completed in detail to allow the evaluation of complementary characters including the complete ornamentation of the antennary basis and the intercoxal sclerites. Despite the fact that they are easily distinguishable from related species, it is not possible to reliably include them in any of the main species-groups of *Eucyclops* and establish their affinities. There are still several taxonomic problems in the genus that certainly deserve further study.

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Redescription of *Acanthocyclops vernalis* (Fischer, 1853) and *Acanthocyclops robustus* (Sars, 1863) from neotypes, with special reference to their distinction from *Acanthocyclops americanus* (Marsh, 1892) and its invasion of Eurasia

Victor R. Alekseev[1,*], Maria R. Miracle[2,†], Maria Sahuquillo[2,3] and Eduardo Vicente[2]

[1] Zoological Institute of the Russian Academy of Sciences, Universitetskaya Embankment 1, St Petersburg 199034, Russia.
[2] Department of Microbiology and Ecology, ICBiBE, University of Valencia, 46100 Burjassot, Valencia, Spain.
[3] Subdirecció General del Medi Natural, Generalitat Valenciana, València, Spain.
† Deceased

* Corresponding author: alekseev@zin.ru

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ABSTRACT

Redescription of *Acanthocyclops vernalis* (Fischer, 1853) and *Acanthocyclops robustus* (Sars, 1863) from neotypes, with special reference to their distinction from *Acanthocyclops americanus* (Marsh, 1892) and its invasion of Eurasia

This paper provides a redescription of *Acanthocyclops vernalis* and *A. robustus* neotypes collected in their type localities in Russia (Orlov pond, Peterhof) and Norway (a stream outcoming from a lake in Oslo) with a special attention to their morphological differentiation from *A. americanus*. Due to overmixing of these species throughout most of the last century and fast arrival and spreading of the aggressive planktonic predator *A. americanus*, all previous indications of *A. robustus* should be checked and confirmed. More than 200 localities in Valencia region (Spain) and more than 30 localities in Armenia, Belgium, Egypt, Finland, France, Kazakhstan, Norway, Russia, Sweden and Tunisia were checked for the presence of *A. americanus*. In all these countries the invader was found in large numbers as dominant species often substituting other native *Acanthocyclops* species in limnetic communities. A brief history and possible explanation of the successful invasion of *A. americanus* in Eurasia is discussed.

Key words: *Acanthocyclops robustus*; *Acanthocyclops vernalis*; *Acanthocyclops* redescriptions; cyclopoid taxonomy; *Acanthocyclops americanus*; biological invasion, distribution in Eurasia

RESUMEN

Redescripción de los neotipos de* Acanthocyclops vernalis *(Fischer, 1853) y* Acanthocyclops robustus *(Sars, 1863), con especial atención a sus diferencias morfológicas con* Acanthocyclops americanus *(Marsh, 1892) y su invasión en Eurasia

Este trabajo proporciona una nueva descripción de los neotipos de A. vernalis *y* A. robustus *recopilados en sus localidades tipo en Rusia (estanque de Orlov, Peterhof) y Noruega (una corriente que sale de un lago en Oslo), con especial atención a su diferenciación morfológica respecto a* A. americanus*. Debido a la mezcla de estas especies prácticamente durante todo el siglo pasado y la rápida llegada del agresivo depredador planctónico* A. americanus*, todas las indicaciones anteriores de presencia de* A. robustus *deben verificarse y confirmarse. Se estudia la presencia de* A. americanus *en más de 200 localidades en la región de Valencia (España) y más de 30 ubicaciones en Armenia, Bélgica, Egipto, Finlandia, Francia, Kazajstán, Noruega, Rusia, Suecia y Túnez. En todos estos países, la especie invasora se encontró en grandes cantidades y como forma dominante, a menudo sustituyendo a las especies nativas de* Acanthocyclops *en las comunidades limnéticas. También se presenta una breve historia y posible explicación de la exitosa invasión de* A. americanus *en Eurasia.*

Palabras clave: Acanthocyclops robustus*;* Acanthocyclops vernalis*; redescripción de* Acanthocyclops*; taxonomía de ciclópidos;* Acanthocyclops americanus*; invasión biológica, distribución en Eurasia*

INTRODUCTION

Acanthocycops americanus (Marsh, 1892) a planktonic cyclopid first described from the Great Lakes in Northern America was found soon after by Lowndes (1926, 1928a) in Great Britain and later on in several other countries of the Old World (Dussart, 1967; Monchenko, 1961; Alekseev & Kosova, 1976). It became the first documented invasive copepod species transported from the Americas to Europe. In the last century it was also found in limnetic communities throughout Eurasia. After Kiefer's (1976) mistaken designation of *A. robustus* (G. O. Sars, 1863) as an older synonym of *A. americanus*, a lot of data indicating their differentiation has been accumulated (Alekseev *et al.*, 2002, Lewis *et al.*, 2004, Alekseev & Monchenko, 2011, Miracle *et al.*, 2013). Clearly visible ecological differences among planktonic *A. americanus* and littoral-benthic *A. robustus* in combination with limitation of classical morphological features used in taxonomy of this cyclopids caused it to become a challenge for several generations of European and American copepodologists.

To solve these taxonomical and ecological problems, representative populations from type localities of the respective taxa were analyzed morphologically and genetically using mitochondrial COI and 12S rRNA markers (Miracle *et al.*, 2013). Molecular-genetic analysis revealed that *A. robustus*, *A. americanus* and *A. vernalis* are well-separated species. All studied European populations of *A. americanus* had small genetic distances to native American reference population obtained from *terra typica*. Consequently, all genetically studied *A. americanus* populations in Eurasia are recognized as a recent biological invasion as it was proposed by Lowndes (1926) (Miracle *et al.*, 2013).

Soon after, a paper appeared on this *robustus*-group, trying to reincarnate wrongly described *A. einslei* and *A. trajani* (Anufriieva *et al.*, 2014). The authors of the paper totally ignored molecular-genetic evidence on the similarity/equivalence of *A. einsli* to *A. robustus* and *A. trajani* to *A. americanus*. They concentrate only on morphological differences quite common in different cyclopid population which is not enough nowadays when molecular-genetic tools have clearly corroborated that the morphologically different populations belong to the same species. Even excellent morphologists in XXI century cannot ignore results of molecular-genetic species validation that was confirmed for this *Acanthocyclops robustus - vernalis* complex recently (Błędzki & Rybak, 2016). A good example could be found in *Cyclops abyssorum* group: many local populations of this group were described as separate species but in direct hybridization experiment of Einsle (1996) easily crossed with each other.

A. americanus dominates in many separated and even isolated waterbodies in Europe except in Arctic areas in Scandinavia and Russia. This species became a main summer planktonic invertebrate predator in large Ukrainian (Dnieper River) and Russian (Don River and Volga River) reservoirs. *A. robustus* should not be a dominating planktonic species due to preference to benthic (littoral) biotopes. All these water-reservoirs appeared during the 50-60s of the last century after creation of cascade of hydropower electric stations. In the Rivers Volga, Dnieper and Dnestr no species of this genus was found in limnetic plankton. *A. americanus* was found in the Caspian Sea, Lake Sevan (Caucasus region), Lake Aral area, Lake Chany and northern reservoirs built at the Yenisey River (Western and Central Siberia). Recently, this species was found in North Africa, the Nile River delta and in Tunisian fresh waters (Victor Alekseev personal data, not published).

At the end of the XIX century, three species of the *Acanthocyclops robustus* group were described variously as: *Acanthocyclops vernalis* Fisher (1853) from the Saint Petersburg area (Russia); *Acanthocyclops robustus* Sars (1892) from the Oslo area (Norway); and *Acanthocyclops americanus* by Marsh (1892) from Wisconsin (USA).

A. americanus was redescribed on the basis of a neotype obtained from *terra typica* (Miracle *et al.*, 2013). At the same time two other closely related sister-species, *A. vernalis* and *A. robustus*, have not yet been redescribed following modern standards. That can create confusion between them and *A. americanus* or some other similar species of *Acanthocyclops*.

This paper provides a redescription of *A. vernalis* and *A. robustus* from the type localities with a special attention to differentiation from *A. americanus*. In addition, we provide new ecological features on *A. americanus* biology and current distribution based, first, on extensive European samplings and, second, on intensive and detailed samplings over a single Mediterranean area, Valencia region, as a case study. Both based on collection from authors and using the morphological differentiation detailed in the first part of the paper. Also, a possible brief history of biological invasion of *A. americanus* in Eurasia is discussed.

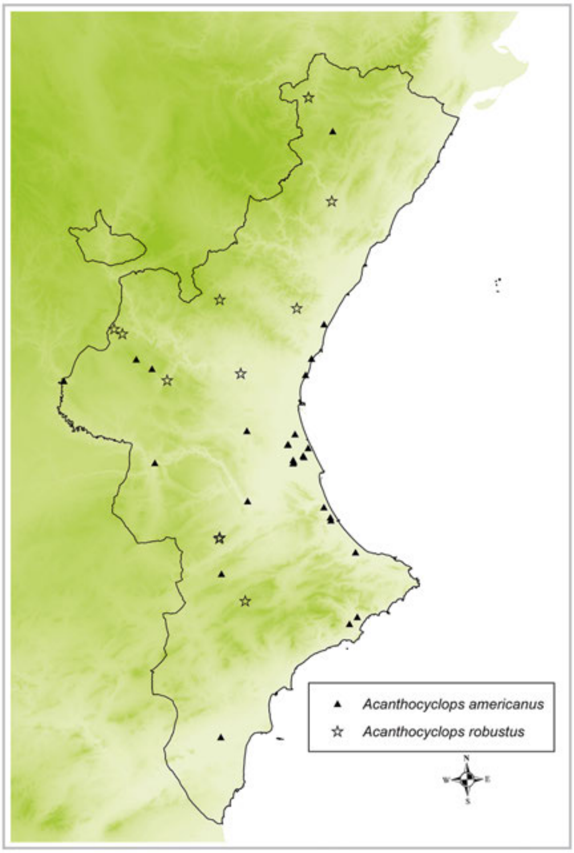

Figure 1. Sampling sites of *Acanthocyclops* species in Valencia region with indication of found species. *Sitios de muestreo de las especies de* Acanthocyclops *en la región de Valencia con indicación de las especies encontradas.*

MATERIALS AND METHODS

1.- Morphological description of *A. americanus*

Morphological description of *A. americanus* had been done on material from *terra typica*, obtained from Prof. Stainly Dodson, Wisconsin USA (Miracle *et al.*, 2013). Hereafter we have used neotype specimens of two morphologically close species which consist of: 10 ♂ and 20 ♀ of *A. vernalis* selected from a sample taken in April 1998 in Orlov pond, Peterhof, St-Petersburg, the type locality for this species as it was described by S. Fischer (1853); 8 ♀ and 2 ♂ of *A. robustus* selected from a sample collected in a stream connected with Lake Maridalsvann, Oslo, Norway. A classical taxonomical method of external morphology examination has been used. Adult females and males before dissecting were photographed from dorsal side for a common view of animal with a 12-megapixel digital photo camera equipping Zeiss ImagerA1 microscope. Then specimens were dissected in a drop of glycerol on a microscope glass covered with a cover glass supported by droplets of plasticine on opposite corners to prevent damage of taxonomically important parts. Drawings were made at 400-1000x magnification with microscope equipped with Nomarski optics and a camera *lucida*, converted to ink, then digitized with scanner (resolution 1200 dpi) and used for mounting in drawings sheet. Each picture had a scale bar added. All slides obtained and used for study were catalogued and placed in the Federal Collection of Zoological Institute of RAS #96-03-16 (Russia)

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Abbreviations used in description and drawings

A1 – antennula; A2 – antenna; CR – caudal rami; CPT – cephalosome; GDS – genital double somite; Enp – endopodite; Exp – exopodite; P1-4 – swimming legs 1-4; P5-6 – rudimental legs; TH1-4 – free thoracic somite

2.- Growth experiments

Experiments and field observation were conducted in the Volga River delta laboratory. In a temporary waterbody placed in Astrakhan State Nature Reserve regularly filled with Volga River water during Spring flooding, we filtered on a daily basis 100 liters with a 10 liter can via an Apstein zooplankton net (mesh size 70 µm) to observe the reactivation of copepod dormant stages present in sediment and population dynamics in several *Acanthocyclops* species coexisting together in the same pool. Species identification was done for females, males and diapausing copepodite stages 4-5 to the last stage. Special taxonomic keys were used for identification (Alekseev, 2000).

For growth experiments, 5-7 female of *Acanthocyclops americanus* and *A. vernalis* with egg sacs were picked up from the pool and incubated in lab until nauplii hatching out. Females were immediately killed and identified to subspecies level. Offspring was used in two types of experiments.

1. In lab conditions 30-50 nauplii of two *Acanthocyclops* species (*A. americanus* and *A. vernalis* as a control) were grown separately (one individual per experimental vial) and measured daily under microscope with ocular micrometer (resolution 7 µm). Each nauplius was placed in 20 ml vial and at copepodite stages they were transferred to 100 ml vials. Water change and organism measurements were done daily at the same time for each animal. Food for nauplii (protists and algae) and for copepodites and adult (crustaceans freshly killed by heating) was replenished after measurements once a day, temperature in lab was checked every 8 hours. Development time for each stage was first converted to 20 ºC and then estimated as an average % from female development time. Metabolic rate and productivity were calculated after Edmondson & Winberg (1972) and also converted to 20 ºC following Crog's evacuation.

2. At least 30 individuals of both species (control groups) were placed in glass tubes covered on both sides with a double mesh membrane and reared directly in pool under natural conditions. Organisms were checked, counted and measured several times until maturation and became reference groups for laboratory grown ones.

Female fecundity was evaluated in about one hundred females randomly collected during the time of the species presence in zooplankton of the temporary waterbody. Embryonic development time was estimated in lab under controlled temperature. Observation for egg hatching time was carried out with a frequency of about one hour.

3.- Distributions: sites and sampling methods

3. 1.- Sampling methods and sites in Valencia region, Spain

First references to the presence of *Acanthocyclops* in Valencia came in the 70s with the study of Albufera lagoon (Blanco, 1976) although unfortunately no specimens are available. Under the supervision of one of us (Dr. Miracle) several studies focused specifically on zooplankton in this coastal lagoon surrounding marshlands and other neighbouring coastal wetlands began in 1982 and have continued, until today (Oltra, 1993; Oltra & Miracle, 1984; Alfonso & Miracle, 1990; Alfonso, 1996; Miracle & Sahuquillo 2002, Moss *et al.*, 2003). The crustacean communities in inland ponds were studied during 2006 and 2007 in extensive surveys to cover the different eco-regions of the Comunitat Valenciana (Sahuquillo & Miracle, 2013). Overall, we sampled 140 ponds (86 rain-fed ponds, 24 stream- or surface water–fed ponds and 30 spring- or groundwater-fed ponds) (Fig. 1). In all cases, to obtain the fullest possible representation of the planktonic and littoral microcrustacean communities in each site, we took semi-quantitative net samples from different mesohabitats (open waters of the central area, the vegetated littoral and very shallow shores). Littoral and plant-associated microinvertebrates were sampled by sweeping a 90 µm hand-net through the vegetated areas and the shallow shores. From open waters, we took samples with 45 µm towing nets. All the material collected was fixed in 4 % formaldehyde and, additionally, some samples were preserved in alcohol for further molecular analyses, when needed.

3.2.- Sampling methods and sites outside Spain

Sites outside Spain where *A. americanus* was found and collected by one of us (V. A.), included 12 countries (Fig. 2). In deep lakes and water reservoirs, for sampling a tow net with open hole

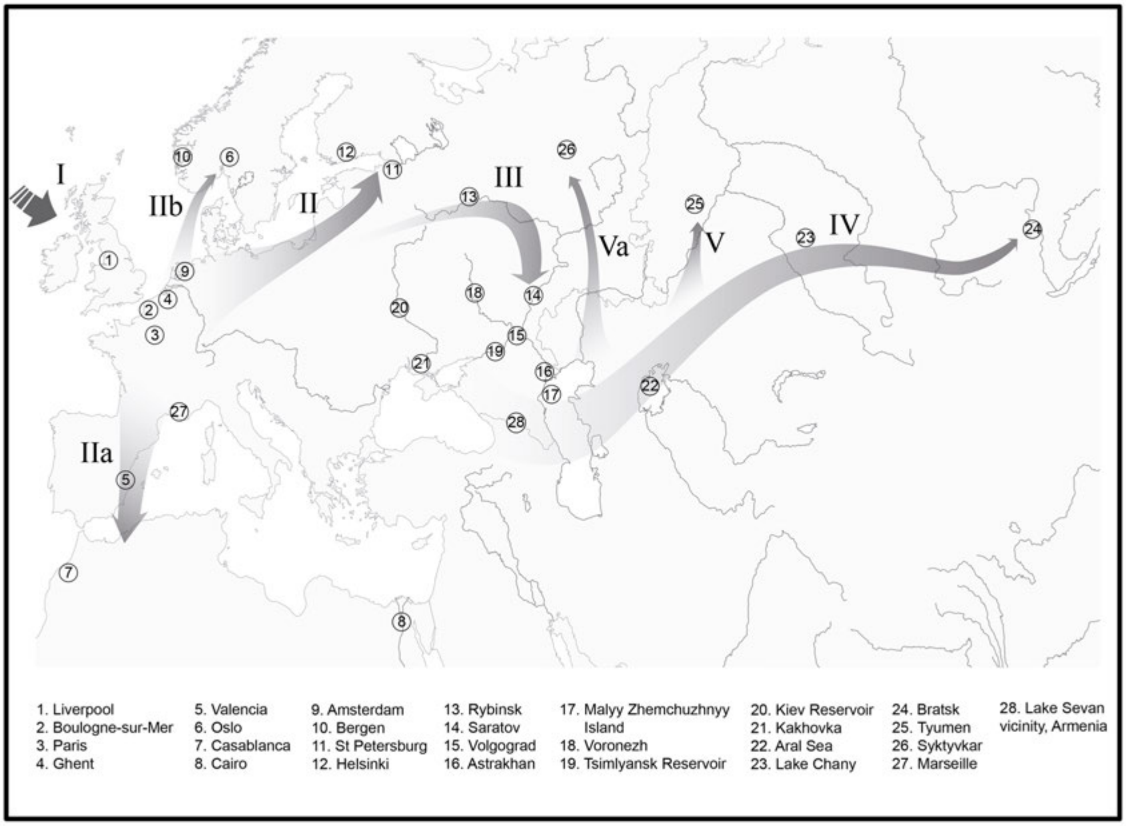

Figure 2. Sampling sites of *Acanthocyclops americanus* found in Eurasia. Numbers indicate the name of the places (see additional information in the Materials and Methods chapter). Arrows indicate the major seasonal aquatic bird migrations. *Lugares de muestreo de* Acanthocyclops americanus *en Eurasia. Los números indican el nombre de los lugares (ver información adicional en el capítulo de Material y Métodos). Las flechas indican las principales rutas estacionales de migración de las aves acuáticas.*

25 cm diameter and mesh size 100 µm was moved from bottom to surface in the central or the deepest parts of lake. In other shallow places, including 3 type localities in Norway, Russia and USA, 50-100 l water with 10 l can via a handle net (mesh size 100 µm) were filtered. Two types of samples were usually collected: formalin conserved for morphological studies and strong alcohol conserved for molecular-genetic and/or morphological studies.

Armenia: 1 shallow lake-let near Lake Sevan, the Caucasus Mountains; Belgium: 3 sites in Ghent and its vicinity; Egypt: 2 sites in Chair vicinity; Finland: 3 sites in Helsinki vicinity; France: 3 sites in Paris, 2 sites in Wimeraux, 3 sites in Camarg, Marseille vicinity; Germany: 3 sites in Plön vicinity; Kazakhstan: 1 site in Aral vicinity; Norway: 2 sites in Oslo vicinity; Russia: more than 20 sites all over the country from St Petersburg in the West to Irkutsk in the East and from Salekhard in the North to Caspian Sea in the South; Sweden: 2 sites in Stockholm vicinity; Tunisia: 2 sites in Hammamet and Tunis; Ukraine: 3 sites in Western, Southern and Eastern parts of the country.

RESULTS AND DISCUSSION

In many localities these three species were found together and in the same place. In large lakes and water reservoirs they maintained different niches as American invasive species is a typical planktonic form while *A. vernalis* and *A. robustus* inhabit littoral areas. Sometimes, as in shallow water bodies like temporary pools in the Volga

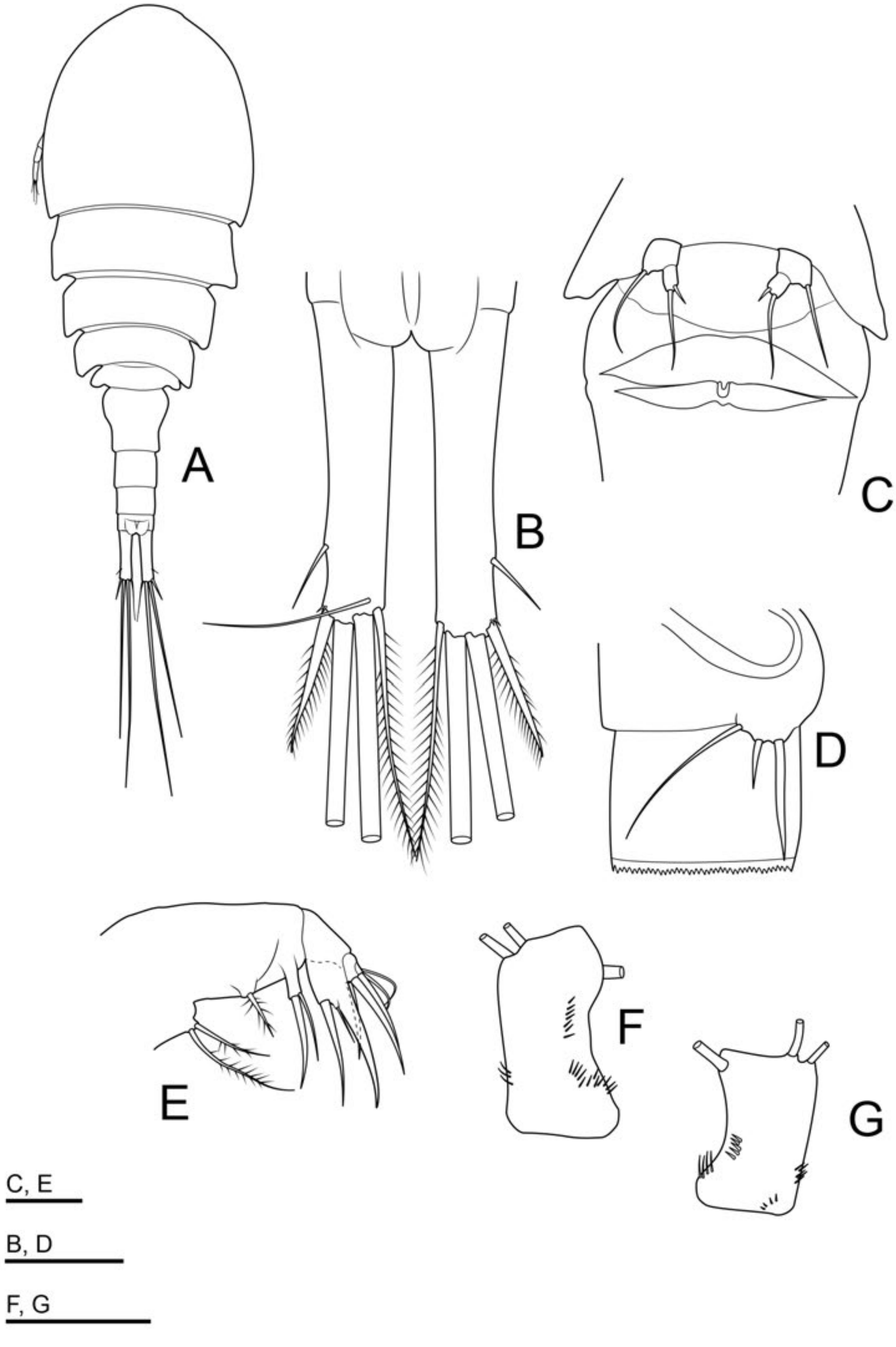

Figure 3. *Acanthocyclops robustus*, holotype of female (A, B, C, E, F, G) and paratype of male (D). Acanthocyclops robustus*, holotipo de hembra (A, B, C, E, F, G) y paratipo de macho (D).*
A - habitus, dorsal; B - caudal rami, dorsal; C - genital segment, ventral; D - P6 ♂; E - maxilla; F - antennal basipodite, frontal; G - antennal basipodite, caudal.
Scale bars: A - 220µm; B, C, D - 50µm; E - 20µm; F, G - 40µm.
Original, V.A.

River delta, they can be found in a sample collected by a handle net among waterplants. Actually, that became one of the reasons why F. Kiefer recognized *A. americanus* as a junior synonym of *A. robustus* after collecting a reference sample in the type waterbody near Oslo (Alekseev *et al.*, 2002). The species being very close to each other show also high variability in classical morphological features.

To avoid the mixing up of these closely related species, hereafter we present descriptions for the three species of the *robustus*-group.

Morphological peculiarities in the *americanus-vernalis-robustus* group

Redescription of *Acanthocyclops robustus* (G.O. Sars, 1863)

(Fig. 3, Fig. 4)

Cyclops robustus Sars, 1863
Acanthocyclops (Acanthocyclops) robustus, Dussart, 1969
Acanthocyclops (s. str.) *vernalis* f. *robusta*, Monchenko, 1974
Acanthocyclops vernalis robustus, Rylov, 1948
Acanthocyclops einslei: Mirabdullayev and Defaye, 2004; Vezhnovets, 2005; Dussart and Defaye, 2006; Rybak and Błędzki, 2010; Blaha, 2010
Acanthocyclops robustus: Kiefer, 1978; Einsle, 1993, 1996; Alekseev, 1995; Mirabdullayev and Defaye, 2004; Dussart and Defaye, 2006; Rybak and Błędzki, 2010, Miracle *et al.*, 2013.

Justification of neotype

Victor Alekseev explored Zoological collections in several locations connected with Sars' work. In Oslo, Lund and Bergen no samples or slides that could be attributed to *A. robustus* were found. Our conclusion is that type material of Sars related to this species does not exist. Lake Maridalsvann is a large waterbody to the north of Oslo practically in the same state. Victor Alekseev visited it in June 2010 and sampled zooplankton in the lake and littoral zone plankton and meiobenthos in the lake and related with its waterbodies: bogs, small rivers coming into the lake. Regarding the plankton in the lake, only *Acanthocyclops americanus* was found in large densities. In littoral areas few members of other *Acanthocyclops* species which could be attributed to *A. robustus* were collected. Finally, in an unnamed stream coming into the lake, only one group of individuals was collected which could be identified as *A. robustus* males, females or copepodite later stages, dark in color, and they clearly different from planktonic form of *Acanthocyclops* from the lake. We used these individuals both for molecular-genetic and morphological studies. The neotype and type group of *A. robustus* were selected from this sample and are described hereafter.

Material examined. The neotype female and paratype male dissected and mounted in glycerol surrounded with Canadian balsam on slides N56736a and N56736b and deposited into the type collection of Zoological Institute of the Russian Academy of Sciences, St. Petersburg, Russia.

Female description
(see Fig. 3, Fig. 4)

Body robust, slightly flattened dorso-ventrally, dark-brown in color. Full body length without caudal setae 1300 µm. Cephalosome as long as wide, with maximum width close to its middle. Lateral angles 5th thoracic segment with winged outgrowths, directed laterally. Genital double-somite about as long as wide, round-shaped, receptaculum seminis as in figure 3. Anal segment with row of small dense spinules, proctodeum with single row of setules on both sides. Caudal rami parallel without hairs on inner or outer margins, 5 times as long as wide. Length proportions of distal setae, beginning from outer terminal seta: 1.0/6.7/9.2/1.7. Inner terminal seta shorter than ramus length. Dorsal seta about 1.2 times as long as outer seta and lateral seta slightly longer than ramus width.

Antennule of 17 segments, not reaching distal margin of cephalothorax. Setation of antennulary segments (aesthetascs in Roman numbers) beginning from first: 8/4/2/6/3+I/1/2/ 1/1/0/1+I/2/0/1/ 2/2+I/7+I. Antenna composed of 1-segmented basipodite bearing exopodite represented by a long seta and the two short setae on opposite side, and a 3-segmented endopodite. Basipodite A2 ornamentation as in figures 3 F and G. Three

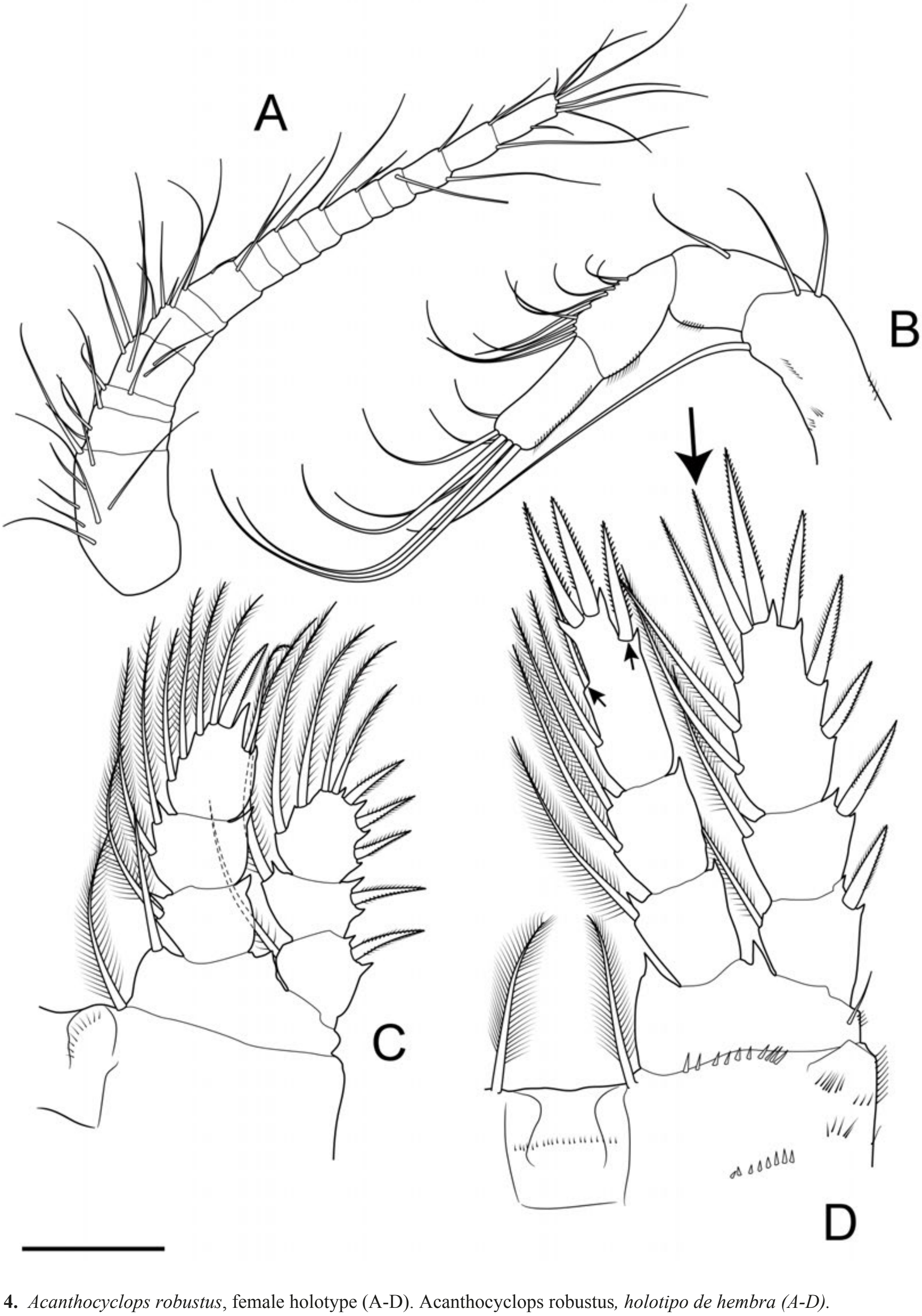

Figure 4. *Acanthocyclops robustus*, female holotype (A-D). Acanthocyclops robustus*, holotipo de hembra (A-D).*
A - antennule; B - antenna; C - P1; D - P4.
Scale bars: A - 200µm; B - 50µm; C, D - 100µm.
Original, V.A.

following segments (endopodite) bearing subsequently: one, nine and seven setae.

Gnathobase of mandible with six strong teeth, rudiment of endopodal segment with 2 long and 1 short seta 1. Maxillula with 3 strong and 3 small teeth, 2 strong setae; maxillulary palp with 7 setae, different in length. Maxilla of 5 segments, praecoxa with 2 strong setae in its middle part; coxa with strong seta in middle and endite bearing 2 claw-like setae; basal endite with 2 very strong claw-like spines, both with row of spinules and small seta near place of fusion of rudimentary endopod. Endopodite 1 with 3 clawlike setae and endopodite 2 bearing distally 2 long setae. Maxilliped of 4 segments, praecoxa + coxa with 2 strong setae in middle part and small setae at their distal end; basis with 2 setae of different length and 3 groups of strong spinules near insertion of setae; first segment of endopodite with strong spine and rudimentary endopod, bearing strong spine and 2 hairless setae.

P1–4 with three-segmented rami. Distal segments of exopodite of legs 1/2/3/4 with 3/4/4/4 spines, respectively. Distal segments of endopodite of legs 1–2 with 1 spine and 5 setae. P4 Enp3 about 2 times as long as wide and inner apical spine 1.13 times longer than outer spine. Lateral outer seta of this segment transformed in strong spine inserted not at same distance from end of segment as inner lateral seta (see Fig. 4D, small arrows). Setae of P4 Exp3 with very short hairs in distal part and long hairs in proximal part (see Fig. 4D, large arrow). P1-3 intercoxal plates with hills and without hairs on its free edge. P4 intercoxal plate with small hills and a row of short denticules in middle part. P4 coxopodite with two rows of spinules near distal and proximal margins and two groups of longer and thinner spinules near lateral margin. Rudimentary P5 two-segmented, basipodite with long outer seta. Distal segment with long apical seta and short inner spine; inner spine slightly more than half of segment length and exceeds segment width.

Male description
(see Fig. 3D and Fig. 7C)

Body length without caudal setae 925 µm. Cephalosome 1.2 times as long as wide, with maximal width close to end of its length. Caudal rami 3.8 times as long as wide, slender inner seta about twice length of outer spine-like seta. Lateral seta placed without spinules in its base. Dorsal seta placed near inner seta insertion, about as long as outer seta. Antennule 12-segmented. Setation of antennulary segments (aesthetascs in Roman numbers) beginning from first: 7(III)/3/1/6(I)/1/1/1/4(I)/0/1/0/7. Antennary basopodite ornamentation as in female. Morphology of mouth appendages and Leg 1–3 basically as in female. Distal segment of P4 Enp about 2,5 times as long as wide, with inner apical spine shorter than segment and 1.15 times as long as outer apical spine. Insertion of lateral seta/spine in this segment as in female. Inner edge of basis of P4 with short hair-setae, coxa of P4 with strong spine. Caudal side of P4 coxa with same groups of spinules as in female but fewer in number. Intercoxal sclerite of P4 without hills and hair-setae on its free edge, but with short row of small spinules in middle. Rudimentary P5 two-segmented, with setae and spine of similar proportion to female. Rudimentary P6 with very short middle seta and very strong inner spine almost reaching length of outer seta (relative lengths 1.0/0.6/0.95) clearly separating it from other species of this group.

Redescription of *Acanthocyclops vernalis* (Fischer, 1853)
(Fig. 5)

Cyclops vernalis Fischer, 1853
Cyclops parcus Herrick, 1882
Cyclops robustus (part.) Lilljeborg, 1901
Cyclops lucidulus G.O. Sars, 1863
Cyclops (Acanthocyclops) vernali Pesta, 1928; Kiefer, 1929; Yeatman, 1944
Acanthocyclops (Acanthocyclops) vernalis Dussart, 1969
Acanthocyclops (s. str.) *vernalis vernalis* Monchenko, 1974
Acanthocyclops (s. str.) *vernalis* f. *robusta* Monchenko, 1974
Acanthocyclops vernalis: Rylov, 1948; (part.) Margalef, 1953; Kiefer, 1978; Einsle, 1993; (part.) Einsle, 1996; Alekseev, 1998; Vezhnovets, 2005; Dussart and Defaye, 2006; Rybak and Błędzki, 2010

Justification of neotype

Cyclops vernalis was described in the middle of XIX century by Fischer from a sample collected in Orlov pond, Peterhof town, St. Peterburg vicinity, Russia. Victor Alekseev checked collections of two Russian Zoological museums that existed at that time: collection of Zoological museum of Imperial Academy of Science in St. Petersburg and collection of Zoological museum of Moscow State University, Moscow. No samples attributed to Fischer were found nor any other materials related to copepods except description on paper. The type Orlov pond at Peterhof still exists and contains all five species described by Fischer including *A. vernalis*. This information let us conclude that type materials of Fischer except description on paper were lost and they do not exist now. Fortunately, the type waterbody, a small Orlov pond continued to exist in the same place all this time and no other *Acanthocyclops* species except *A. vernalis* were found

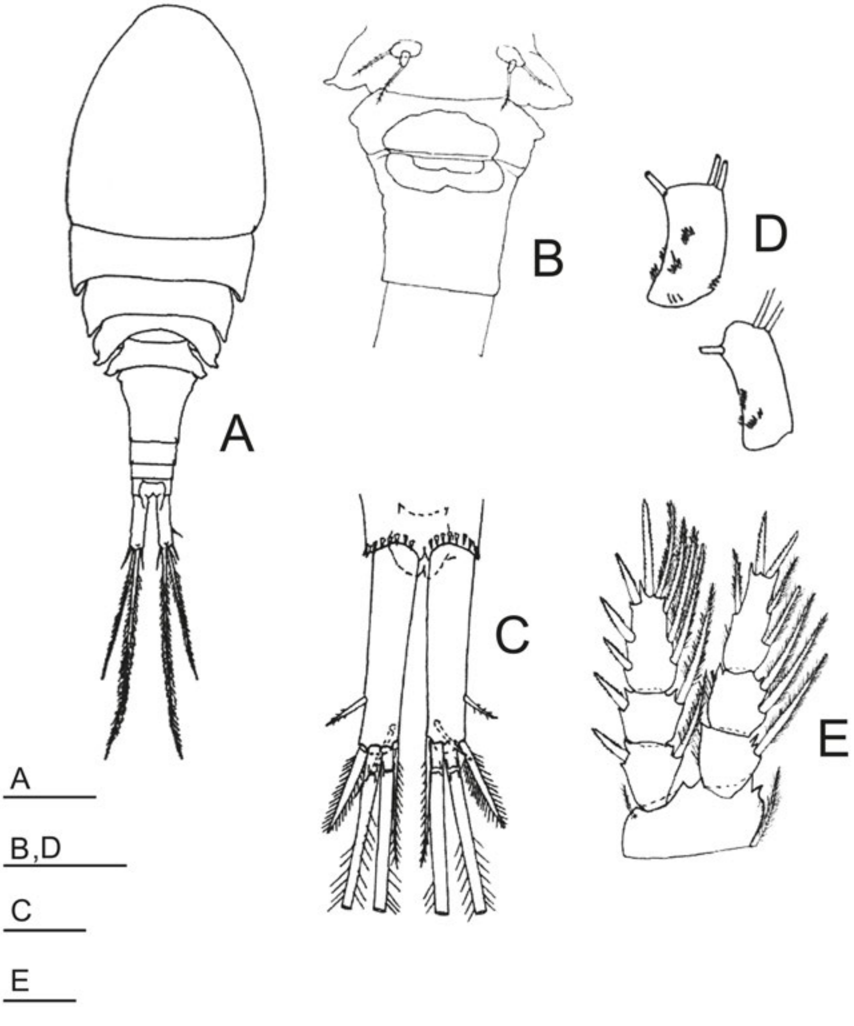

Figure 5. *Acanthocyclops vernalis*, holotype of female. Acanthocyclops vernalis*, holotipo de hembra.*
A - habitus, dorsal; B - genital segment, ventral; C - caudal rami, ventral; D - antennal basipodite, frontal; D1 - antennal basipodite, caudal; E - P4.
Scale bars: A - 200µm; B - 100µm; C - 50µm; D, D1 - 25µm; E - 50µm.
Original, V.A.

after its observation in April 1998 and all following visits up to now, approximately one-two times a year. That grants us the splendid possibility to establish the neotype of *A. vernalis* with a high probability from the same population that Fischer worked on.

Material examined. The neotype female and paratype male dissected and mounted in glycerol surrounded with Canadian balsam on slides N56736 and N56737 and deposited into the type collection of Zoological Institute of the Russian Academy of Sciences, St. Petersburg, Russia. The type series also includes two females and one male preserved in pure glycerol.

Female description

(see Fig. 5)

Body transparent, colorless. Full body length without caudal seta 1375 µm. Genital double-somite approximately as long as wide, with angular anterior part and sharpened lobes, receptaculum seminis with an anterior depression. Caudal rami parallel or slightly divergent, without hairs on inner margin, 5.5 times as long as wide. Inner seta 1.15 times longer than outer seta. Dorsal seta shorter than outer seta. Lateral setae not reaching ramus length. Length proportions of distal setae, beginning from outer terminal seta: 1/8/12/1.3.

Antennule 17-segmented almost reaching distal margin of cephalosome. Setation of antennulary segments (aesthetascs in Roman numbers) beginning from first: 8/4/2/6/3+I/1/2/1/1/0/1+I/2/0/1/2/2+I/7+I. Shortest setae of distal segment less than length of distal segment. Antenna composed of 1-segmented basipodite bearing exopodite represented by a long seta and the two short setae on opposite side, and a 3-segmented endopodite. Basipodite A2 ornamentation as in figure 5. Three following segments (endopodite) bearing subsequently: one, nine and seven setae.

Gnathobase of mandible with six teeth, rudiment of endopodial segment with 2 long setae and 1 short seta. Maxillula with three strong and three small teeth, two strong setae; maxillar palp with seven setae, different in length. Maxilla of five segments, praecoxa with two strong setae in its middle part; coxa with strong seta in the middle and an endite bearing two claw-like setae; basal endite with two very strong claw-like spines, both with a row of spinules and small setae near the place of fusion of the rudimentary endopod. Endopodite 1 with three clawlike setae and endopodite 2 bearing distally two long setae. Maxilliped of four segments, praecoxa + coxa with two strong setae in the middle part and small setae at their distal end; basis with two setae of different length and three groups of strong spinules near the insertion of the setae; first segment of endopod with strong spine and rudimentary endopod, bearing a strong spine and two hairless setae.

Swimming legs 1–4 with three-segmented rami. Distal segments of endopodite of Legs 1–2 with one spine and five setae. Distal segment of endopod Legs 3–4 elongated, with two strong spines at its end. In P4 Enp3 2.9 times as long as wide and outer apical spine slightly longer than inner one (1.2 times). Insertion of the lateral outer seta not at the same level from the end of the segment as the second lateral inner seta. Outer lateral seta of this segment not transformed in spine as it was in previous species, all setae homogeneously pinnated. Intercoxal plate of P4 with small hills and row of denticules in middle. P4 coxopodite with row of spinules along proximal margin, two groups of spinules at distal margin and groups of spinules near lateral margin. Rudimentary P5 two-segmented, basal segment with long outer seta. Distal segment with long apical seta and short inner spine less than half of segment length with few spinules at the base.

Male description

(see Fig. 5G)

Body length without furcal seta 915 µm. Cephalosome 1.3 times as long as wide, with maximal width close to end of its length. Caudal rami 4.3 times as long as wide, slender inner seta more than twice length of outer spine-like seta. Lateral seta placed without spinules in its base. Dorsal seta placed near inner seta insertion, about 1.2 times longer than outer seta. Antennule 12-segmented. Setation of antennular segments (aesthetascs in Roman numbers) beginning from first: 7(III)/3/1/6(I)/1/1/1/4(I)/0/1/0/7. Antennal baso-

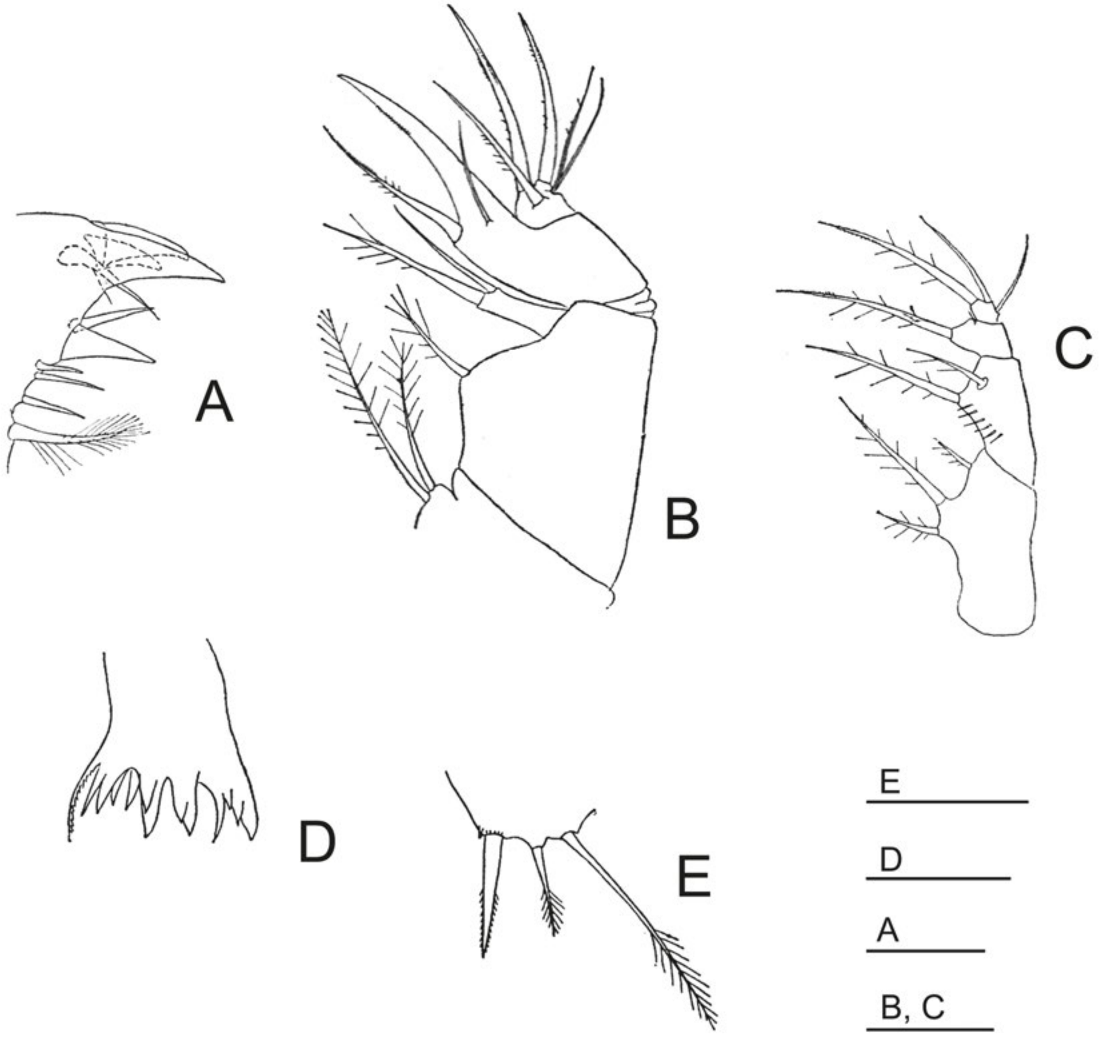

Figure 6. *Acanthocyclops vernalis*, female from the type seria (A-D), male (E). Acanthocyclops vernalis*, hembra del tipo seria (A-D), macho (E).*
A - gnathobase of maxilla; B - maxilla; C - maxiliped; D - mandibula, distal part; E - male, rudimentary leg 6.
Scale bars: A, D, E - 30µm; B, C - 50µm.
(After Monchenko, 1973 with changes)

podite ornamentation as in female. Morphology of mouth appendages and Leg 1–3 basically as in female. Distal segment of endopod Leg 4 about three times as long as wide, with inner apical spine about as long as the segment and 1.25 times as long as outer apical spine. Insertion of lateral seta in this segment as in female. Intercoxal sclerite Leg 4 without hills and hair-setae on its free edge, but with short row of small spinules in middle. Rudimentary Leg 5 two-segmented, with setae and spine of similar proportion to female. Rudimentary P6 with strong inner spine, middle seta shorter than spine and a very long outer seta (relative lengths 1.0/0.6/1.4).

Detailed redescription of *A. americanus* neotype from the type locality was done in our previous study (Miracle *et al.*, 2013). Hereafter we decided to briefly remind the most important characters to show as the greatest possible difference in morphology among these three species.

Acanthocyclops americanus (Marsh, 1892)
(Fig. 6)

Cyclops viridis americanus Marsh, 1892
Cyclops americanus Marsh, 1920
Acanthocyclops americanus f. *spinosa* Monchenko, 1961: Vezhnovets, 2005
Acanthocyclops robustus f. *limnetica* Petkovski, 1975
Acanthocyclops robustus: (part.) Kiefer 1976; Fryer, 1985; Dodson, 1994; Lescher-Moutoué, 1996; Einsle, 1996; Dussart and Defaye, 2006; Rybak and Błędzki, 2010
Acanthocyclops trajani Mirabdullayev and Defaye, 2002; Dussart and Defaye, 2006; Rybak and Błędzki, 2010; Blaha, 2010
Acanthocyclops (Acanthocyclops) americanus: Dussart,1969
Acanthocyclops americanus: Rylov, 1948; Alekseev, 1995; Vezhnovets, 2005; Alekseev *et al.*, 2002

Female. Body colorless, length 1100-1600 µm. Egg sacs with large number of transparent small size eggs. Cephalosome as narrow ellipsoid with maximal width close to middle. Fifth segment without long wings laterally. Genital double-somite round-shaped in lateral sides, 1.1-1.3 times as long as wide, with seminal receptacle with wide transparent zone in its frontal part (see Fig. 7). Caudal rami parallel or divergent, 4.5-6.5 times as long as wide. Innermost seta sub-equal to caudal rami length. Antenna composed of 1-segmented basipodite bearing exopodite represented by a long seta and the two short setae on opposite side, and a 3-segmented endopodite. Basipodite A2 ornamentation as in figure 6D,E. Mouth appendages

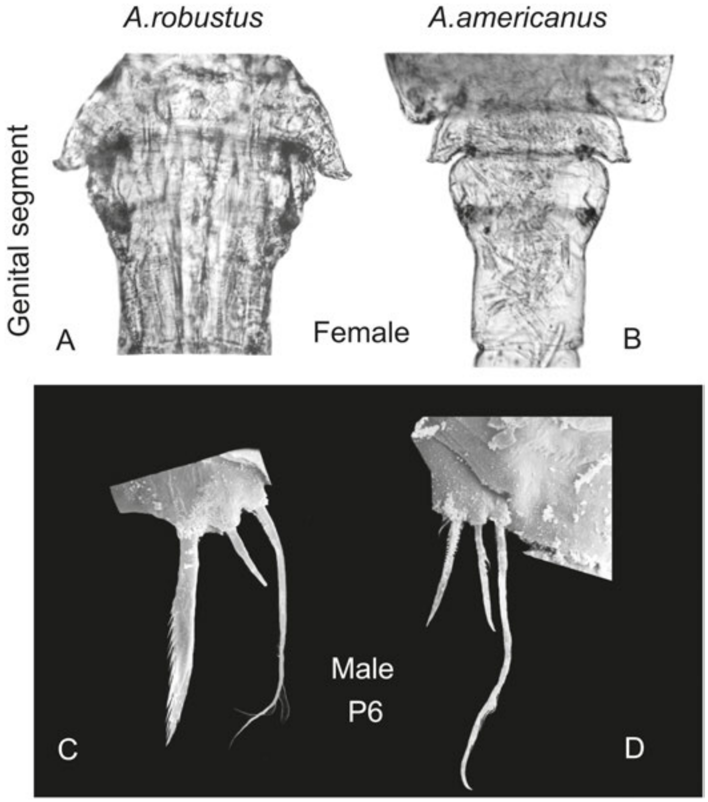

Figure 7. Comparative illustration showing the clear differences between female (A, B) and male (C, D) of *Acanthocyclops robustus* (A, C) and *Acanthocyclops americanus* (B, D). *Ilustración comparativa que muestra las claras diferencias entre la hembra (A, B) y el macho (C, D) de* Acanthocyclops robustus *(A, C) y* Acanthocyclops americanus *(B, D).*

as in other *Acanthocyclops* species.

P4 Enp3 2.6 times as long as wide; inner apical spine 1.1 times as long as outer spine and longer than segment itself. Insertion of the lateral outer seta approximately at the same level from the end of the segment as the second lateral inner seta. Intercoxal sclerite Leg 4 with small hills and row of small denticules in middle part. Coxa Leg 4 with four groups of denticles typical for several species of *robustus*-group. Distal segment exopod Leg 4 with distal seta longer than nearest distal spine. Rudimentary Leg 5 two-segmented, basal segment with long outer seta. Distal segment with long apical seta and short inner spine less than half of segment length.

Male. Body length without caudal seta 900-1100 µm. Cephalosome 1.3 times as long as wide, with maximal width close to end of its length. Caudal rami 4-5 times as long as wide, slender inner seta more than twice length of outermost spine-like seta. Lateral seta placed without spinules in its base. Dorsal seta placed near innermost seta insertion, about 1.2 times longer than outer seta. Antennule 12-segmented. Antennal basopodite ornamentation as in female. Distal segment of endopod Leg 4 about 2.5 times as long as wide, with inner apical spine about as long as the segment and 1.25 times as long as outer apical spine. Insertion of lateral seta/spine in this segment as in female. Rudimentary Leg 5 as in female. Rudimentary Leg 6 with inner weak spine, middle seta slightly shorter than spine and a very long outer seta 2 times longer than inner spine. (Fig. 7D).

Comments on *robustus*-group differentiation

Several morphological differences among the specimens from the type localities were found to assign the specimens unambiguously to the mentioned species of *Acanthocyclops* (Table 1).

Male differences in P6 construction among these species is also very clear and stable as observed in many populations inhabiting Eurasia (Alekseev & Monchenko, 2011). In *A. americanus* male inner spine is weak and similar in size to middle seta when outer seta is very long and twice of inner spine length (Fig. 7D). In *A. robus-*

Table 1. The most important morphological features in female that separate invasive *A. americanus* from native sister species in Eurasia. *Características morfológicas más importantes en hembras para separar la especie invasora* A. americanus *de las especies nativas de Eurasia.*

Characters/species	*A. americanus*	*A. vernalis*	*A. robustus*
Body shape and color	*Elongated, colorless*	*Robust, yellowish*	*Flattened, dark brown*
Cephalosome L/Wratio	*>1*	*1*	*1 or <1*
Caudal rami shape and L/W ratio	*Divergent*	*Slightly divergent*	*Parallel*
CR innermost/ outer seta ratio	*>2*		*1.7*
GDS shape and L/W ratio	*round laterally >1*	*Angular laterally =1*	*round laterally =1*
P4 End3 distal seta inserted places	*At the same distances from end*	*At different distances from end*	*At different distances from end*
P4 End3 L/W ratio	*More than 3*	*2.5 - 3*	*Less than 2.5*
P4 End3 inner distal spine/L	*More than 1*	*About 1*	*Less than 1*
P4 Exp3 distal seta / distal spine	*Seta>spine*	*Subequal*	*Seta<spine*

tus this spine is strong and twice of middle seta length and subequal to outer length (Fig. 7C). In *A. vernalis* these appendages are of intermediate size and length (see Fig. 5). As mentioned above for females differences in P4 distal segment construction are similar to males and can be used for species separation purposes.

Microcharacters of the antennary basipodite that are so good taxonomic characters for species discrimination in some genus of cyclopoids such as *Eucyclops* or *Mesocyclops*, in our opinion, are too variable at population level in the genus *Acanthocyclops* to be used for species separation, at least in the *robustus* group (Lewis *et al.*, 2004; Alekseev, 2015).

The critical point that lets us separate Eurasian species from the invasive American species became molecular genetic assessment conducted by Maria Rosa Miracle (Miracle *et al.*, 2013). This study also improved species synonymy. *Acanthocyclops americanus* from Wisconsin (USA) and *A. trajani* described from Etàng de Noes (France) were morphologically and genetically alike as the same happening to *A. robustus* and *A. einslei* Mirabdullayev & Defay, 2004 (Alekseev & Monchenko, 2011; Miracle *et al.*, 2013).

Key for species identification in *robustus*-group

Females

1. P4 End3 inner distal seta and lateral seta inserted at the different levels from the segment end .... *A. robustus* (G.O. Sars, 1863) [Native species within the Palearctic Region in Spain in rivers and springs (Maria Miracle, unpublished)]
\- P4 End3 inner distal seta and lateral seta inserted at the same level from the segment end 2
2. P4 End3 apical inner spine shorter than apical outer spine .... *A. vernalis* (Fischer, 1853) [Native species within the Palearctic Region but missing in Spain (probably)]
\- P4 End3 apical inner spine equal or slightly longer than apical outer spine .... *A. americanus* (Marsh, 1892)

Males

1. Rudimental P6 with weak inner spine; outer seta twice of its length .... *A. americanus* (Marsh, 1892)
\- This spine is strong; outer seta shorter 2
2. Inner spine in P6 about as long as outer seta and twice of middle seta .... *A. robustus* (G. O. Sars, 1863)
\- Inner spine significantly shorter .... *A. vernalis* (Fischer, 1853)

Distribution of *A. americanus* and *A. robustus* at Valencia Region, eastern Spain

Long time uncertainty of identification within the *robustus*-group caused by biological invasion of *A. americanus* in Eurasia resulted in wrong determination of species in most European countries (except Russia and Ukraine) and an absence of relevant information on these two species interaction as well as real areal occupied by *A. americanus* in XX century. Only recently, after molecular-genetic confirmation, regarding the invasive origin of *A. americanus* in Europe, this situation begins to turn better but still needs improving (Alekseev & Monchenko, 2011; Miracle *et al.*, 2013).

The morphological characters selected by us here in separating *A. americanus* from the native Eurasian species *A. robustus* were hereafter used to reveal modern distribution of *A. americanus* and *A. robustus* as well as the coexistence of these species in Valencia area, Spain. Thus, this becomes the first detailed study so far on the problem in Europe.

A database of the presence of these two *Acanthocyclops* species occurring in the Valencia region (eastern Iberian Peninsula) was compiled from several extensive sampling surveys, carried out by the authors under direction of Dr. Miracle through several different research projects from 2000 to 2017. In total we collected data for more than 200 sampled sites. Sampled sites included: coastal lagoons (with low marine influence) and their surrounding temporary marshes along the Valencia coast; small ponds fed by rain (temporary ponds) or springs (permanent ponds) and sites associated with fluvial courses and major rivers.

Table 2. Characteristics of wetlands where *Acanthocyclops* species have been found in Valencia region from a total of more than 200 sites studied. *Características de los humedales en los que se encontraron especies de* Acanthocyclops *en la Comunitat Valenciana de un total de más de 200 lugares estudiados.*

Type of sites	*A. americanus* (Number of sites, %)	*A. robustus* (Number of sites, %)	Conductivity (µS/cm)
Coastal lagoons and marshes	8	0	1800 - 3000
River ponds	5	4	538 - 1330
Permanent ponds	12	0	300 - 3050
Temporary ponds	2	10	93 - 585
TOTAL	27 (66%)	14 (34%)	

Acanthocyclops species were found in 41 sites (Table 2). They were never found together in the same aquatic habitat and none of these species has been found in brackish waters. The distribution of both species highlights some of their differences in ecology. *A. americanus* was the most frequent species found in the current survey. In accordance with the data available in the studied area, *A. robustus* has not been found in any coastal lagoon, neither in permanent ponds, where was possibly substituted by the other species. It is important to note that the most permanent ponds sampled were also located in coastal areas. Ponds associated with rivers harbors both species approximately in equal proportions. The greatest part of sites with *A. robustus* are small temporary ponds isolated from water courses. From our sampling experience, these two species have been seen to occupy different habitats: *A. robustus* showing littoral preferences and *A. americanus* being more pelagic. Spatially (see Fig. 1) most sites with *A. robustus* are located in inland areas. This distribution possibly suggests that in the more connected coastal areas with greater anthropogenic influence, the invasive *A. americanus* species has displaced the native *A. robustus* species, while the latter is only preserved in small isolated ponds and in rivers in the most unpopulated inland area.

Other countries

In most sites outside Spain these two species were also found separately with two exceptions that became important for our study as we explain in the history of biological invasion of *A. americanus* and reasons why it was so successful.

The first exception – the type locality of *A. robustus*. In the type for *A. robustus* lake in Oslo (Norway) *A. americanus* was dominant in plankton when *A. robustus* was found in small numbers in a small shallow stream out-coming from the lake (Alekseev & Monchenko, 2011). This sampling, done at the end of June 2010, confirms our previous inference that in the 60s Kiefer also sampled *A. robustus* in the same lake and getting them together came to the mistaken conclusion on their identity (Alekseev *et al.*, 2002). As a result he down-graded *A. americanus* as a synonym of a younger *A. robustus*. That mistake in effect blocked studies on the *A. americanus* invasion in Palearctic for more than 50 years. Hereafter we try to restore the main elements of the history of biological invasion of this species in Eurasia.

The second exception – seasonally temporary water bodies in the River Volga delta. The spring filling temporary water bodies in the Volga delta are highly productive and short time (1-3 months) existing habitat where species competition is low due to extremal environmental time limitation. This lets many species to co-exist effectively producing dormant stages highly resistant to desiccation and in the same site up to 200 copepod species including all three from the robustus group can be found living together (Alekseev, 1980). These special conditions are very suitable to study and compare life-cycle parameters in these morphologically close sister-species.

A history of biological invasion of *A. americanus*

According to literature data in native habitat (the Americas) a planktonic cyclopid *Acanthocyclops* morphologically close to *A. vernalis* and *A. robustus* was described from the Great Lakes in North America (Marsh, 1892). Soon after, it was found in U. K. (Lowndes, 1928) possibly after oversea transport with ship ballast waters (Alekseev *et al.*, 2002). In native habitat it is also known as a planktonic form, often dominating in eutrophic water bodies in Canada and Mexico (V. A. personal data).

In Spain and in France it was found in the middle of the last century (Dussart, 1967). As *A. robustus* f. *pelagica* it was registered in Hungary (Petkovski, 1975). Also it was presented in Kiefer's sample collected at south Norway (Oslo) (see Fig. 2) where it co-existed with *A. robustus*. This co-existence of *A. americanus*, dominating the plankton of the lake, and *A. robustus*, in low densities inhabiting small rivers connected with the lake, was confirmed by V. A. sampling at the same site in 2010.

It was a dominant species in Belgian creeks and in ponds of the Central City Park in Ghent, whereas *A. robustus* was only found in one location, a roadside ditch (Alekseev *et al.*, 2003).

V. Rylov a very accurate taxonomist did not report any *A. americanus* in Russia before 1941 but included it in his key for cyclopids of USSR as a potential invader (Rylov, 1948). So we can suppose that *A. americanus* was not in Russia at that time as well as in European continental countries it was only found in the middle of last century.

Consequences after creation of large limnetic habitats (cascades of hydro-electric power stations with water reservoirs) on the Volga, Don, Dnepr and Dnestr Rivers

Acanthocyclops americanus was found for the first time in Russia in Rybynsk water reservoir (on the River Volga, started on 1941, and finished in 1947) in 1957 (Prof. A. Monakov personal communication to V.A.), then in Ukraine in Kievskoye reservoir (on the River Dnepr) and in Dnestr reservoir (Monchenko 1961, 1974). After creating a cascade for the hydro-power stations on the River Volga (1950-1960s) this species was found first in Saratov reservoir, then in Volgagrad reservoir (Vijushkova, 1962). In both reservoirs *A. americanus* was a dominative cyclopid in summer zooplankton (Vijushkova, 1962). In 1974 this species was also found in high densities in temporary waterbodies in the River Volga Delta (Alekseev & Kosova, 1975). Few years later *A. americanus* was the only planktonic crustacean reported in the Caspian Sea (sea lagoons and rain pools on the island Zhemchuzhniy) (Chuykov, 1986). It was also present in Gulf of Finland (the Baltic Sea near St. Petersburg), 1981; in Armenia, near Lake Sevan (Caucasus), 1990; in Kazakstan near Lake Aral; and in lake Chanu (1985), in Western Siberia (a lake near the Tola River, Tumen, 1987); in Central Siberia, Bratsk water reservoir (the River Enisey), 1988. In 2015 it was found in a temporary rain pool near Syktyvkar (the northern Ural Mountains), and the same year in Hanko island near Helsinki (Finland). In 1996 and in 2003 it was found in Egypt (Nile river near Cairo). Recently it was also indicated in the Lena River delta (Abramova *et al.*, 2017). Most of the lines connecting the sampling sites where *A. americanus* was found are similar to flyways of aquatic birds seasonal migrations.

Biology of *Acanthocyclops americanus*, an invasive species in the Volga versus *vernalis-robustus* native species

Life cycle parameters that include growth and productivity rates, egg size and fecundity in *A. americanus* was compared to the same rates in *A. vernalis* obtained at the same temperature and food conditions (Alekseev, 1980). The invasive species had shorter maturation time (about 12 % faster at 20 ºC), higher metabolic level as well as feeding and productivity rates. If we compare time from egg laying until dormant stage (Copepodid 4), *A. americanus* show times shorter than those for *A. vernalis*. All these biological features along with higher fecundity are thought to be responsible for this invasive species to increase its competitive edge and substitute similar sized sister species in their niches.

Acanthocyclops americanus seasonality in the River Volga delta

This species was found in highest density not in permanent water bodies (rivers, lakes etc.) but in temporary ones (Alekseev & Kosova, 1976). In spring river flooding pools that filled with water every year and are used by local and migrating fish for breeding and short time feeding, all these three species coexist together in more or less equal proportions (Alekseev, 1980). They appear at the very first stage of the spring water flooding.

There was some regularity in the appearance of these species at 4th copepodid stages immerged in wet or just covered with thin films of water soil after 9-11 month dormancy in dry conditions.

A. vernalis appears first, then in one week comes *A. robustus* and finally when water temperature reaches 18-20 ºC *A. americanus* are found among reactivated formerly dormant copepods. In few days copepodites become adults (males in 1-3 days earlier) and immediately start reproducing. Before fish hatching the first generation of tiny nauplii appear and very soon they substitute adults consumed by young fish. Being numerous and small they can escape complete elimination by countless fish. Before the drying up of the water bodies, when fish are gone, populations of these species are ready for dormancy at copepodite 4th stage thus they finish their life cycle having only one or two generations in a season. Even in these ephemeral water bodies we can observe differences in ecological demands between the invasive (*Acanthocyclops americanus*) and native (*A. vernalis* and *A. robustus*) species (Alekseev, 1980).

Appearance times for these species in the temporary water bodies were related to water temperature. At the very beginning of spring, when river water just arrived in this pool and temperature was about 14 ºC, *A. vernalis* copepodite stages were found emerging from the soil and in few days became adults and started breeding. At 16 ºC, copepodite *A. robustus* appeared and turned to maturation and breeding. Soon after, at 18-20 ºC, *A. americanus* and *A. americanus spinosus* had appeared. *A. americanus* seems to be more sensitive to temperature at the beginning of the life cycle and that may become a limiting factor in distribution of the species in the Arctic zone. Climate warming, on the other hand, must be a favorable factor to overcome this obstacle and to increase areal distribution northwards.

Small egg size and significantly higher female fecundity in invasive species could give them competitive advantages but only in high trophic conditions. If food is limited, especially in naupliar stages of development, species with large egg size will survive better. So the native species are deemed to be more effective in the early spring time and late fall season whereas invasive species should be more competitive in early summer time when small sized prey such as protist and rotifers are numerous.

Other studies on this species showed other advantages such as omnivorous diet (Sarma *et al.*, 2019), rapid population growth rates, higher fecundity, effective predation on other cyclopid species and use of aquatic birds for dispersal in a similar way to Cladocerans (Ślusarczyk *et al.*, 2019). Also the naupliar stages outcompete herbivorous cladocerans and thus possibly are dominant in zooplankton communities (Enríquez-García *et al.*, 2013). We think that the fast and wider distribution of *A. americanus* became possible due to all mentioned above biological features.

CONCLUSIONS

1. The putative invasive species *Acanthocyclops americanus* can now be easily separated from the two native similarly sized species *A. vernalis* and *A. robustus*, both using morphological characters and molecular techniques. The most important taxonomical differences in females are related with P4 structure, in males with P6 armament.

2. Study on copepod diversity carried out in about 200 sites in Valencia region (Spain), revealed the presence of *Acanthocyclops* species in 41 sites: in 66 % of the sites the genus is represented by the putative invasive species *A. americanus* and only in 34 % sites *A. robustus* was the species found. These two species were never found together at the same place.

3. Analysis of literature and our own sampling data on distribution of *A. americanus* in Eurasia lets us assemble a possible story of its invasion begun in early XX century from U.K.; today, the species is distributed throughout the Palearctic Region.
4. Experimental study of life cycle in *A. americanus* and the two native species showed a competitive advantage of the invasive planktonic species in limnetic communities in high trophic conditions.
5. Eutrophication, creation of large limnetic habitats (water reservoirs) and climate warming were favorable factors for the great success of its invasive and colonizing abilities to dominate in such a huge territory within a century.
6. There is a high risk that *A. americanus* in the near future may expand its area to the Arctic zone due to climate warming. As it was observed in many cases in shallow water bodies, *A. americanus* became the dominant planktonic species. Arctic water bodies are mainly small and shallow with limited number of invertebrate species. *A. americanus* as a very effective predator may dramatically change these vulnerable ecosystems. The last finding of *A. americanus* in the Arctic zone (Comi Republic and North Urals) recently sampled by E. Fefilova (personal communication) confirmed these preliminary conclusions.

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Winter-Spring fauna of Cladocera of Dali Bai Autonomous Prefecture, Yunnan Province, China

Artem Y. Sinev [1,2,*], Yangliang Gu [3] and Bo-Ping Han[3]

[1] Biological Faculty, M.V. Lomonosov Moscow State University, Leninskie gory, Moscow 119991, Russia.
[2] A. N. Severtsov Institute of Ecology and Evolution, Leninsky Prospect 33, Moscow 119071, Russia.
[3] Department of Ecology and Institute of Hydrobiology, Jinan University, Guangzhou 510632, China..

[*] Corresponding author: artem.sinev@gmail.com

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ABSTRACT

Winter-Spring fauna of Cladocera of Dali Bai Autonomous Prefecture, Yunnan Province, China

The Cladoceran fauna of Dali Bai Autonomous Prefecture, Yunnan Province, China was investigated in January 2015 and April 2016. Water bodies studied include Lake Erhai, second largest lake in Yunnan, several large lakes and reservoirs, and numerous smaller water bodies, located at 1800-2000 m above sea level. The winter-spring fauna of the area included 34 species, most of them Palearctic or widely distributed species, with plus five predominantly Paleotropical taxa. Thirty-one species occurred in Lake Erhai, while species richness in other water bodies not exceeded 14 species. Only parthenogenetic females were found in the majority of species, suggesting that Dali area climate is mild enough to allow Palearctic species to overwinter as active populations. *Ilyocryptus cuneatus* Śtifter, 1988 is recorded for China for the first time; *Alona kotovi* Sinev, 2012, *Camptocercus uncinatus* Smirnov, 1971 and *Disparalona ikarus* Sinev & Kotov, 2011, are also first records for continental China.

Key words: Cladocera, zoogeography, morphology, China, Yunnan

RESUMEN

Fauna invernal y primaveral de cladóceros de la prefectura autónoma de Dali Bai, provincial de Yunnan, China

Durante enero de 2015 y abril de 2016 se investigó la fauna de cladóceros de la prefectura autónoma de Dali Bai, provincia de Yunnan, China. Las masas de agua estudiadas incluyeron el lago Erhai, segundo en tamaño de Yunnan, varios lagos grandes y embalses, y numerosos pequeños cuerpos de agua, situados a una altitud de 1800 – 2000 msnm. La fauna invernal y primaveral de cladóceros incluye 34 especies, muchas de ellas de distribución Paleartica o ubiquistas, con sólo cinco taxones encontrados de predominancia Paleotropical. Se encontraron 31 especies en el lago Erhai, mientras que la riqueza en otras masas de agua no superó las 14 especies, mostrando la mayor diversidad de biotopos en este lago. Sólo se encontraron hembras partenogenéticas de la mayoría de las especies, lo que sugiere que la zona de Dali tiene un clima suficientemente suave como para permitir a las especies paleárticas pasar el invierno como poblaciónes activas. Ilyocryptus cuneatus *Śtifter, 1988 se ha citado en China por primera vez;* Alona kotovi *Sinev, 2012,* Camptocercus uncinatus *Smirnov, 1971 y* Disparalona ikarus *Sinev & Kotov, 2011, se han citado también por primera vez para la China continental.*

Palabras clave: *cladóceros, zoogeografía, morfología, China, Yunnan.*

INTRODUCTION

The Cladocera of East Asia have been actively investigated during the last decades. Local faunas were recently studied in Far East of Russia (Kotov *et al.*, 2011ab), South Korea (Kotov *et al.*, 2012, 2017; Jeong *et al.*, 2014) and China (Sinev *et al.*, 2015; Chertoprud *et al.*, 2017) and numerous new taxa were described from the region (Kotov *et al.*, 2006; Korovchinsky, 2009; Kotov & Sinev 2011; Jeong *et al.*, 2012, 2013, 2017; Sinev *et al.*, 2016) Checklists were published for Korea (Jeong *et al.*, 2014) and China (Xiang *et al.*, 2015; Ji *et al.*, 2015). But most regions of continental China are not yet well studied, with most faunistic data for the country based on ecological work with doubtful identifications (Xiang *et al.*, 2015; Ji *et al.*, 2015).

The main water body of Dali Bai Autonomous Prefecture (Yunnan Province, China) is Lake Erhai, the second largest lake in Yunnan Province. It is a fault lake situated at 1972 m above sea level. Its area is about 250 square kilometers; length of the lake is about 40 kilometers and width is about 7–8 kilometers; average depth is 11 m. Several large natural lakes - Xihu, Zibi, Jainhu, Chenghai and Shuizhangdi, as well as numerous smaller reservoirs and artificial ponds are located in the vicinity of Lake Erhai at altitudes of 1800-2000 m. Lake Erhai region is rather insular, surrounded by mountainous ranges with no lentic water bodies. Distance from Lake Erhai to Lake Dianchi (Kunming Area), the largest lake in the region, is over 250 km, with no natural lakes in between, but several small reservoirs were constructed there in the course of the 20th century.

The Cladoceran fauna of the area has been poorly studied; most data come from previous hydrobiological studies of Lake Erhai (Lu, 1939). They revealed 12 species of Cladocera there. Pelagic communities of Lake Erhai were investigated on a regular basis (Li *et al.*, 1963; Wu & Wang, 1999; Yang *et al.*, 2014), and Cladocera remains in the lake sediments were also studied recently (Liu *et al.*, 2014; Lu *et al.*, 2016). During the last century, Lake Erhai became subject to eutrophication, exotic fish introduction and degradation of natural macrophyte communities, lead to strong changes in cladoceran species composition (Liu *et al.*, 2014; Lu *et al.*, 2016). No data for other large water bodies in Dali area are available. While records for Yunnan cladocerans in general are numerous (see Xiang *et al.*, 2015; Ji *et al.*, 2015), only few records refer specifically to Dali Area.

The aim of present study was to evaluate spring-winter fauna of Cladocera of Lake Erhai and other water bodies of Dali area (Table 1), with special attention to the littoral fauna.

MATERIALS AND METHODS

Samples were collected by the two first authors in January 2015 and in April 2016 by a standard plankton net (25 cm diameter, 100 µm mesh size) and dip net (100 µm mesh size) and immediately fixed in 3 % formaldehyde. In locations with a complex structure of the littoral zone, samples were taken in different zones of vegetation, i.e. plants with submerged or floating leaves were rinsed by water to a bucket, reed-like plants scraped by dip nets, etc. Specimens were selected from the samples under a binocular stereoscopic microscope Biolam MBI-10, placed on slides in a drop of a glycerol-water mixture and studied under microscopes Olympus CX-41 and CX-51. Dissections were conducted by electrolytically sharpened tungsten needles. Measurements were conducted using an eyepiece-micrometer. Drawings were made by means of camera lucida.

RESULTS

We recovered 34 species of Cladocera, including a single species of Sididae, 11 species of Daphnidae, a single species of Macrothricidae, two species of Ilyocryptidae, a single species of Bosminidae, and 18 species of Chydoridae (see Table 2). *Ilyocryptus cuneatus* Śtifter, 1988 is here recorded for China for the first time. Three more species, *Alona kotovi* Sinev, 2012, *Camptocercus uncinatus* Smirnov, 1971 and *Disparalona ikarus* Sinev & Kotov, 2011, known only from Hainan Island (Sinev *et al.*, 2015) are recorded for continental China for the first time. Comments on the taxa are represented below. Among 34 species recorded, 7 are planktonic and 27 are

Table 1. List of sampling sites (W - winter samples, S - spring samples). *Lista de lugares de muestreo (W – en invierno, S – en primavera).*

	Sampling site	Latitude	longitude	Date
W1	Lake Erhai, Xi'er river mouth within Dali city, stone embankment, abundant submerged macrophytes and filamentous algae	N 25º 36' 11.4"	E 100º 13' 43.9"	19-01-2015
W2	Lake Erhai, stone embarkment within Dali city, abundant submerged macrophytes and filamentous algae	N 25º 36' 26.7"	E 100º 14' 27.8"	19-01-2015
W3	Decorative pond within Dali city, with rock bottom without macrophytes	N 25º 36' 33.9"	E 100º 15' 08.8"	19-01-2015
W4	Lake Erhai, low clay shore, submerged terrestrial grasses	N 25º 39' 53.2"	E 100º 17' 01.9"	19-01-2015
W5	Lake Erhai, open rocky littoral, filamentous algae on stones	N 25º 39' 52.9"	E 100º 16' 52.6"	19-01-2015
W6	Lake Erhai, littoral zone with water hyacinth (*Eichhornia crassipes)*	N 25º 41' 27.2"	E 100º 16' 26.6"	19-01-2015
W7	Lake Erhai, open rocky littoral, filamentous algae on stones,	N 25º 44' 00.7"	E 100º 13' 56.3"	19-01-2015
W8	Lake Erhai, rocky littoral with water caltrop (*Trapa* sp.) and filamentous algae on rocks	N 25º 49' 53.2"	E 100º 13' 09.9"	19-01-2015
W9	Lake Erhai, low clay shore, submerged terrestrial grasses	N 25º 51' 11.9"	E 100º 13' 14.9"	19-01-2015
W10	Lake Erhai, rocky littoral with water caltrop (*Trapa* sp.), water hyacinth (*Eichhornia crassipes)*, and filamentous algae on rocks	N 25º 53' 43.1"	E 100º 12' 52.0"	19-01-2015
W11	Lake Erhai, low clay shore with water hyacinth (*Eichhornia crassipes)* and water fern (*Salvinia* sp.)	N 25º 57' 09.9"	E 100º 09' 24.1"	19-01-2015
W12	Paddy field	N 25º 56' 10.5"	E 100º 06' 30.8"	19-01-2015
W13	Temporary pool with submerged terrestrial vegetation and water fern (*Salvinia* sp.) on meadow close to W12			19-01-2015
W14	Ornamental pond with stone embankment, without macrophytes	N 25º 51' 13.0"	E 100º 13' 35.4"	19-01-2015
W15	Irrigation ditch in garlic field	N 26º 00' 41.0"	E 100º 04' 25.8"	20-01-2015
W16	Lake Xihu, littoral zone with water hyacinth (*Eichhornia crassipes*) and abundant submerged macrophytes	N 26º 00' 56.1"	E 100º 03' 07.5"	20-01-2015
W17	Lake Xihu, littoral zone with abundant submerged macrophytes	N 26º 00' 57.5"	E 100º 03' 09.7"	20-01-2015
W18	Pond near Lake Xihu, overgrown with water lily and submerged macrophytes	N 26º 00' 54.5"	E 100º 03' 12.4"	20-01-2015
W19	Pond near Lake Xihu, overgrown with submerged macrophytes, close to W18	-	-	20-01-2015
W20	Paddy field	N 26º 00' 57.7"	E 100º 03' 13.2"	20-01-2015
W21	Lake Zibi, stone embankment, abundant submerged macrophytes	N 26º 08' 04.5"	E 99º 57' 20.3"	20-01-2015
W22	Lake Zibi, low coast, submerged terrestrial grasses	N 26º 08' 04.1"	E 99º 57' 08.1"	20-01-2015
W23	Fish pond close to Lake Zibi, abundant filamentous algae	N 26º 08' 05.3"	E 99º 57' 07.2"	20-01-2015
W24	Yuhua reservoir, low coast with submerged terrestrial grasses	N 26º 25' 35.4"	E 99º 57' 54.0"	20-01-2015
W25	Lake Jainhu, shallow littoral zone with submerged macrophytes	N 26º 29' 06.4"	E 99º 56' 36.9"	20-01-2015
W26	Pond in agricultural area, without macrophytes	N 25º 43' 26.3"	E 100º 10' 54.6"	21-01-2015
W27	Fish pond in agricultural area, without macrophytes	N 25º 43' 42.3"	E 100º 10' 59.6"	21-01-2015
W28	Lake Erhai, submerged terrestrial vegetation and water hyacinth (*Eichhornia crassipes)*	N 25º 43' 51.8"	E 100º 11' 14.4"	21-01-2015
W29	Lake Erhai, rocky littoral with water hyacinth (*Eichhornia crassipes*) and submerged macrophytes	N 25º 44' 00.0"	E 100º 11' 04.6"	21-01-2015
W30	Irrigation channel going from Lake Erhai, about 50 m from the lake, muddy bottom and submerged macrophytes	N 25º 44' 15.3"	E 100º 10' 18.6"	21-01-2015
W31	Reservoir, clay shore with submerged terrestrial grasses	N 25º 27' 16.2"	E 100º 11' 42.8"	21-01-2015
W32	Chenguanchang reservoir, clay shore without macrophytes	N 25º 24' 19.8"	E 100º 11' 18.5"	21-01-2015
W33	Pool in river valley	N 25º 24' 12.2"	E 100º 12' 05.6"	21-01-2015
S1	Pond near Lake Xihu, overgrown with water lily and submerged macrophytes (same loc. as W18)	N 26º 00' 54.5"	E 100º 03' 12.4"	19-04-2016
S2	Lake Xihu, littoral zone with abundant submerged macrophytes	N 26º 00' 53.7"	E 100º 03' 11.4"	19-04-2016
S3	Lake Xihu, littoral zone with water hyacinth (*Eichhornia crassipes*) and abundant submerged macrophytes (same loc. as W16)	N 26º 00' 56.1"	E 100º 03' 07.5"	19-04-2016

Cont.

Table 1. (cont.)

S4	Lake Xihu, littoral zone with abundant submerged macrophytes (same loc. as W17)	N 26º 00' 57.5"	E 100º 03' 09.7"	19-04-2016
S5	Lake Zibi, littoral zone with abundant submerged macrophytes	N 26º 08' 22.5"	E 99º 57' 07.7"	19-04-2016
S6	Lake Zibi, littoral zone with abundant submerged macrophytes	N 26º 08' 23.2"	E 99º 57' 05.2"	19-04-2016
S7	Lake Zibi, littoral zone with abundant submerged macrophytes	N 26º 08' 35.8"	E 99º 58' 58.9"	19-04-2016
S8	Pond near Lake Zibi, abundant submerged macrophytes	N 26º 08' 37.4"	E 99º 56' 58.0"	19-04-2016
S9	Lake Zibi, littoral zone with reed stands	N 26º 08' 50.5"	E 99º 56' 55.6"	19-04-2016
S10	Reservoir, clay shore	N 26º 17' 24.5"	E 99º 58' 39.7"	19-04-2016
S11	Yuhua reservoir, low clay coast with filamentous algae	N 26º 25' 26.2"	E 99º 58' 02.0"	19-04-2016
S12	Lake Jainhu, shallow littoral zone with submerged macrophytes (same loc. as W25)	N 26º 29' 06.4"	E 99º 56' 36.9"	20-04-2016
S13	Lake Jainhu, open shallow littoral close to S12	-	-	20-04-2016
S14	Reservoir, clay bottom with submerged terrestrial vegetation	N 25º 28' 34.9",	E 100º 25' 40.2"	20-04-2016
S15	Fish pond	N 25º 27' 06.7"	E 100º 34' 06.6"	20-04-2016
S16	Fish pond	N 25º 26' 11.7"	E 100º 35' 14.7"	20-04-2016
S17	Southern coast of Shuizhangdi lake, abundant submerged macrophytes and filamentous algae	-	-	20-04-2016
S18	Pond close to Shuizhangdi lake, abundant submerged macrophytes	N 25º 26' 15.4"	E 100º 36' 03.1"	20-04-2016
S19	ditch with concrete embankment close to Shuizhangdi lake, abundant submerged macrophytes	N 25º 26' 17.2"	E 100º 36' 04.7"	20-04-2016
S20	Shuizhangdi lake, abundant submerged macrophytes and filamentous algae	N 25º 26' 20.7"	E 100º 35' 36.7"	20-04-2016
S21	Lotus pond	N 25º 26' 19.8"	E 100º 35' 33.1"	20-04-2016
S22	Reservoir, sample at the dam	N 25º 41' 39.4"	E 100º 38' 49.2"	20-04-2016
S23	Same reservoir as S22, clay bottom without macrophytes	N 25º 41' 13.3"	E 100º 38' 43.2"	20-04-2016
S24	Small reservoir, abundant submerged macrophytes,	N 26º 23' 34.6"	E 100º 38' 38.7"	21-04-2016
S25	Same reservoir as S24, open littoral with clay bottom	N 26º 23' 34.1"	E 100º 38' 25.9"	21-04-2016
S26	Lake Chenghai, open gravel littoral	N 26º 27' 49.6"	E 100º 38' 44.2"	21-04-2016
S27	Lake Chenghai, open gravel littoral	N 26º 33' 34.1"	E 100º 38' 27.4"	21-04-2016
S28	Pool in stream bed	N 26º 05' 23.2"	E 100º 33' 47.0"	21-04-2016
S29	Pond connected by channel to Lake Erhai	N 25º 43' 16.9"	E 100º 11' 32.1"	22-04-2016
S30	Pond in agricultural area, without macrophytes (same loc. as W26)	N 25º 43' 26.3"	E 100º 10' 54.6"	22-04-2016
S31	Fish pond without macrophytes (same loc. as W27).	N 25º 43' 42.3"	E 100º 10' 59.6"	22-04-2016
S32	Lake Erhai, rocky littoral with water hyacinth (*Eichhornia crassipes*) and submerged macrophytes, (same loc. as W29).	N 25º 44' 00.0"	E 100º 11' 04.6"	22-04-2016
S33	Lake Erhai, shallow littoral zone with water caltrop (*Trapa* sp.) and abundant submerged macrophytes	N 25º 56' 22.3"	E 100º 05' 50.5"	22-04-2016
S34	Irrigation channel close to Lake Erhai, abundant submerged macrophytes	N 25º 56' 11.4"	E 100º 06' 32.3"	22-04-2016
S35	Lake Erhai, Xi'er river mouth within Dali city, abundant submerged macrophytes and filamentous algae (same loc. as W1)	N 25º 36' 11.4"	E 100º 13' 43.9"	22-04-2016
S36	Lake Erhai, stone embankment within Dali city, abundant submerged macrophytes and filamentous algae (same loc. as W2)	N 25º 36' 26.7"	E 100º 14' 27.8"	22-04-2016
S37	Decorative pond within Dali city, with rock bottom without macrophytes (same loc. as W3)	N 25º 36' 33.9"	E 100º 15' 08.8"	22-04-2016
S38	Lake Erhai, open rocky littoral, filamentous algae on stones (same loc. as W5)	N 25º 39' 52.9"	E 100º 16' 52.6"	22-04-2016
S39	Lake Erhai, bay separated by stone pier, abundant submerged macrophytes, filamentous algae on stones	N 25º 41' 22.8"	E 100º 16' 24.8"	22-04-2016
S40	Lake Erhai, open rocky littoral, filamentous algae on stones (same loc. as W7)	N 25º 44' 00.7"	E 100º 13' 56.3"	22-04-2016

substrate-associated. We did not find *Bosmina coregoni*, which is present in pelagic zone of Lake Erhai across the year according to Yang *et al.* (2014), probably because we collected only shore samples.

In winter, most frequent littoral species in the area were *Chydorus* cf. *sphaericus* and *Flavalona costata*. Other common species were *Disparalona chappiuisi*, *Camptocercus uncinatus* and *Coronatella rectangula*. In spring, all these species remain common, *Chydorus* cf. *spahaericus* and *Coronatella rectangula* being most frequent, and two more species, *Simocephalus mixtus* and *Pleuroxus quasidenticulatus* became common as well. Among planktonic species, *Bosmina longirostris* was most common during both winter and spring.

Family Sididae

Sida ortiva Korovchinsky, 1979. Littoral species, associated with vegetation; specimens are able to attach to a substrate. Common in the area in both winter and spring, found in vegetated areas of Lake Erhai, lake Xihu and Lake Jianghu, Zibi reservoir and in ponds. East Asian species, distributed from East Siberia to Bangladesh and South Vietnam (Korovchinsky, 2004). A first record for Yunnan province. Earlier records of *Sida crystallina* (O. F. Müller, 1776) from South-East China, including Yunnan (Chiang & Du, 1979) probably all belong to *S. ortiva* (see Korovchinsky, 2004). For description, see Korovchinsky (2004), and Kotov *et al.*, (2012).

Family Daphniidae

Ceriodaphania dubia Richard, 1894. Planktonic species, found only in Lake Erhai in winter (loc. W2, W6, W7). Widely distributed Palearctic species, rare in China, with few records in Taiwan, Wuhan, Jiangxi, Yunnan (Xiang *et al.*, 2015), and in Xinjiang (Chertoprud *et al.*, 2017). Studied specimens (Fig. 1A-C) share diagnostic characters of Palearctic populations. For description of European populations, see Hudec (2010).

Ceriodaphnia quadrangula (O.F. Müller, 1785). Planktonic species, rare in the area, found in Erhai in winter and spring (loc. W11, W12, S29) and in Zibi in spring only (loc. S7). Widely distributed Palearctic species, common in China (Xiang *et al.*, 2015). Recorded for Dali Area by Lu (1939). For description of European populations, see Hudec (2010)

Ceriodaphnia reticulata (Jurine, 1820). Planktonic species. Found only in Lake Chenghai in spring. Widely distributed Palearctic species, rare in China, recorded mostly in mountainous areas (Tibet, Xinjiang, Yunnan, Gansu and Qinghai provinces), and in Nanjing, Jiangsu (Xiang *et al.*, 2015) but, in our opinion, the latter record is doubtful. For description of European populations, see Hudec (2010).

Daphnia (Daphnia) galeata Sars, 1864. Pelagic planktonic species, found in Lake Erhai, Lake Xihu, and Lake Shuizhangdi, in open water samples. Common Palearctic species. According to Xiang *et al.* (2015), common in large lakes and reservoirs of China, including Yunnan, and almost the only pelagic *Daphnia* in Southern China, reaching the island of Hainan in the South. For Yunnan, taxonomic status of *D. galeata* from Lake Dianchi was confirmed by genetical studies (Ma *et al.*, 2015). Record of *D. hyalina* from Erhai (Lu, 1939) probably belongs to *D. galeata* instead. For description, see Benzie (2005).

Daphnia (Daphnia) pulex Leydig, 1860. Planktonic species, inhabiting small water bodies and vegetated coastal areas. Rare in the area, found in winter only in Erhai (loc. W1) and Xihu (loc. W16), both locations are characterized by abundant submerged macrophytes. Palearctic species, so far recorded mostly in North and Central China, records in South-East China rare (see Xiang *et al.*, 2015). For description, see Benzie (2005).

Daphnia (Ctenodaphnia) sinensis Gu, Xu, Li, Dumont, Han, 2013. Planktonic species inhabiting mostly small water bodies. Rare in the area, found in winter in one locality in Lake Erhai (loc. W2). Species is widely distributed in Asia and Africa (see Popova *et al.*, 2016), was recorded from China as *D. similis* and *D. similoides*, both these species are not present in the region (Popova *et al.*, 2016). For description, see Popova *et al.* (2016).

Scapholeberis kingi Sars, 1888. Littoral species associated with water surface. Found in a single locality, a pond in agricultural area Lake

Table 2. Distribution of cladocera species in Dali area in winter 2015 and spring 2016. Abbreviations: W, winter samples; S, spring samples; EastAs, East Asian species; PaleoTr, Paleotropical; PanTr, Pantropical; PaleAr, Palearctic; SC, probable species-complex; WD, widely distributed or cosmopolitan. *Distribución de las especies de cladóceros en la zona de Dali en invierno de 2015 y primavera de 2016. Abreviaturas: W, muestras de invierno; S, muestras de primavera; EastAs, especies del Este asiático; PaleoTr, Paleotropical; PanTr, Pantropical; PaleAr, Paleártica; SC, probable complejo de especies; WD, especie cosmopolita o ampliamente distribuida.*

	Area of distribution	Erhai		Xi Hu		Zibi		Jianhu		Chenghai	Shuizhangdi	Small resrvoirs		Ponds		Samples total	Samples total
Time of sampling		W	S	W	S	W	S	W	S	S	S	W	S	W	S	W	S
Sida ortiva	EastAs	+	+	+		+	+	+						+	+	7	7
Ceriodaphnia dubia	PaleAr	+														3	0
Ceriodaphnia quadrangula	PaleAr	+	+				+									2	2
Ceriodaphnia reticulata	PaleAr									+						0	1
Daphnia galeata	PaleAr	+	+	+					+		+	+	+			7	4
Daphnia pulex	PaleAr	+														2	0
Daphnia sinensis	WD	+														1	0
Scapholeberis kingi	PaleoTr													+	+	1	1
Simocephalus congener	WD	+														2	0
Simocephalus expinosus	WD	+														1	0
Simocephalus mixtus	WD	+	+	+		+	+				+	+		+	+	9	14
Simocephalus serrulatus	WD	+	+	+		+	+				+				+	4	9
Macrothrix spinosa	PaleoTr	+														1	0
Ilyocryptus spinifer	WD	+														3	0
Ilyocryptus cuneatus	PaleAr												+			0	1
Alona affinis	PaleAr		+											+	+	1	2
Alona guttata	WD, SC	+	+				+					+		+	+	4	3
Alona kotovi	EastAs	+														3	0
Anthalona harti	PaleoTr	+														1	0
Camptocercus uncinatus	PaleAr	+	+	+	+	+	+						+			10	10
Coronatella rectangula	PaleAr	+	+	+	+		+	+			+	+	+	+	+	11	23
Flavalona costata	PaleAr	+	+	+	+	+	+	+				+	+	+	+	20	17
Graptoleberis testudinaria	WD, SC	+	+		+	+	+								+	3	8
Leydigia ciliata	PaleoTr		+							+						0	2
Oxyurella tenuicaudis	PaleAr	+														1	0
Alonella excisa	WD, SC	+	+					+								2	1
Chydorus cf. *sphaericus*	WD, SC	+	+	+	+	+	+	+	+	+	+	+		+	+	27	25
Disparalona chappuisi	PaleoTr	+	+	+	+	+	+	+	+					+	+	10	15
Dispralona ikarus	EastAs		+										+			0	2
Pleuroxus aduncus	PaleAr	+	+		+										+	2	5
Pleuroxus laevis	PaleAr	+														5	0
Pleuroxus quasidenticulatus	PaleoTr	+	+	+	+		+			+			+	+	+	6	11
Pseudochydorus bopingi	EastAs	+	+				+			+			+			3	6
Bosmina longirostris	WD, SC	+	+		+	+	+	+	+			+	+		+	11	10
Total number of species		31		14		14		8		5	5	11		13			

Erhai in both winter and spring (loc. W26, S30). Paleotropical species, described from Australia (Dumont & Pensaert, 1983). Common in China (Xiang *et al.*, 2015). For description, see Dumont & Pensaert (1983).

Simocephalus (Echinocaudus) congener (Koch, 1841). Littoral species, associated with vegetation; specimens are able to attach to substrate. Several specimens found in Lake Erhai (loc. W9) in winter. Species inhabit Central and Eastern Europe and Siberia (Orlova-Bienkowskaya, 2001). Recorded for China only in Potatso National Park in Shangri-La (Xiang *et al.*, 2015) and in Manning reservoir on Hainan Island (Sinev *et al.*, 2015). This species is close to *S. expinosus*. According to Orlova-Bienkowskaya (2001), it is a separate species which differs from *S. expinosus* by the morphology of the postabdominal claw, having a pecten of 18–22 spines on the outer side instead of 8–12 in *S. expinosus*. However, in reality, such conclusions must be checked, as the species diagnosed by Orlova-Bienkowskaia (2001) is open to criticism (Huang *et al.*, 2014).

Simocephalus (Echinocaudus) expinosus (De Geer, 1778). Littoral species, associated with vegetation; specimens are able to attach to substrate. Rare in the area, few specimens found in Lake Erhai (loc. W29) in winter. Distributed in West Hemisphere (Orlova-Bienkowskaia, 2001); for list of locations in China see Xiang *et al.* (2015). For description, see Orlova-Bienkowskaia (2001).

Simocephalus (Simocephalus) mixtus (O. F. Müeller, 1776). Littoral species, associated with vegetation; specimens are able to attach to substrate. Most common species in the area, found in vegetated areas of lakes and ponds in both winter and spring. Widely distributed species, recorded in all climatic zones of North Hemisphere (Orlova-Bienkowskaia, 2001), common on Hainan Island (Sinev *et al.*, 2015), but in continental China recorded only once, in Heilongjiang Province (Xiang *et al.*, 2015). Possibly confused with its sibling-species, *S. vetulus* and *S. vetuloides*, and being identification based on the key by Orlova-Bienkowskaia (2001) vulnerable to criticism (Huang *et al.*, 2014).

Simocephalus (Coronocephalus) serrulatus (Koch, 1841). A littoral species, associated with vegetation; specimens capable to attach to the substrate. Common species in the area, found in vegetated areas of lakes and ponds in both winter and spring. Cosmopolitan species, common in the Oriental region (Orlova-Bienkowskaia, 2001), widely distributed but rare in China (Xiang *et al.*, 2015). For a description see Orlova-Bienkowskaia (2001).

Family Ilyocryptidae

Ilyocryptus cuneatus Śtifter, 1988. Benthic, associated with muddy or clayey bottom. Rare in the area, found in a single small reservoir in spring (loc. S23). Palearctic species; in Asia recorded for Asian Russia and Japan (Kotov & Śtifter, 2006). The first record for China, and also southernmost record for the species. Distinctive characters of species includes retained valves from previous molt (Fig. 1D); setae on posterior margin of valves with distinctive spine-like setules one side in its basal portion (Fig. 1E); postabdomen with anus opening at the middle (Fig. 1F); preanal margin of postabdomen with doubled spines and no setules near their bases (Fig. 1G); and postbadominal claw with long ventral setules at the base (Fig. 1F). For detailed description, see Kotov & Śtifter (2006).

Ilyocryptus spinifer Herrick, 1882. Benthic, associated with mud or clay bottom. Rare in the area, found only in winter in Lake Erhai (W7, W11) and in a temporary pool in agricultural area (W13). No evidence of the non-cosmopolitanism of this species has been found (Kotov & Dumont, 2000); common in the Oriental region, including China. For list of records in China, see Xiang *et al.* (2015). For detailed description see Kotov & Dumont (2000) and Kotov *et al.* (2012) for Far Eastern populations.

Family Macrothricidae Norman & Brady, 1867

Macrothrix spinosa King, 1853. Littoral species, associated with vegetation. Rare in the area; several specimens found in Lake Erhai in winter (loc. W2). Species presumed to be Pantropical (Smirnov, 1992), common in the Oriental region. Common in South-East China (Ji *et al.*, 2015).

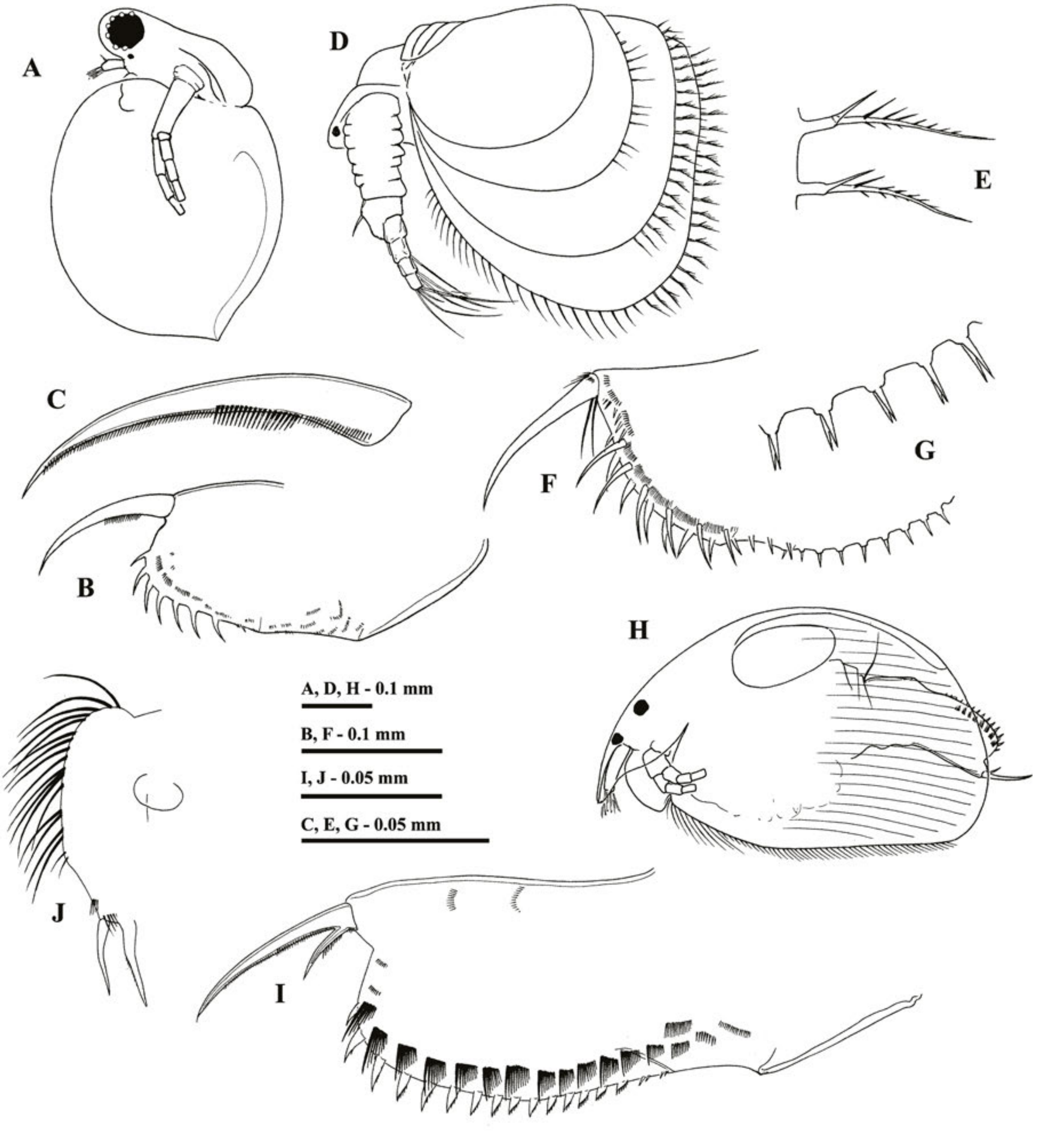

Figure 1. A-C, *Ceriodaphania dubia* Richard, 1894 from Lake Erhai (loc. W2), parthenogenetic female. A, lateral view. B, postabdomen. C, postabdominal claw. D-G, *Ilyoryptus cuneatus* Śtifter, 1988 from reservoir in Dali area (loc. S 23), parthenogenetic female. D, lateral view. E, setae of posterior margin of valves. F, postabdomen. G, spines on preanal margin of postabdomen. H-J, *Alona kotovi* Sinev, 2012 from Lake Erhai (loc. W11), parthenogenetic female. H, lateral view. I, anterior margin of thoracic limb I. Ceriodaphania dubia *Richard, 1894 del lago Erhai (loc. W2), hembra partenogenética. A, vista lateral B, postabdomen. C, pinza postabdominal. D-G,* Ilyoryptus cuneatus *Śtifter, 1988 del embalse en la zona de Dali (loc. S 23), hembre partenogenética. D, vista lateral. E, seta del margen posterior de las valvas. F, postabdomen. G, espines del margen preanal del postabdomen. H-J,* Alona kotovi *Sinev, 2012 del lago Erhai (loc. W11), hembra partenogenética. H, vista lateral. I, margen anterior del limbo torácico I.*

For description, see Smirnov (1992) and Hollwedel *et al.* (2003); for description of Indochina populations see Idris (1983).

Family Chydoridae, subfamily Aloninae

Alona affinis (Leydig, 1860). Littoral species, inhabiting both vegetated and open littoral zone. Rare in the area, found in decorative pond in Dali city in both spring and winter (loc. W3, S37), and in one location in Lake Erhai in spring (S35). Common widely distributed Palearctic species, also recorded in South Africa (Sinev, 2009). Common in China, recorded for Yunnan (Ji *et al.*, 2015). For description, see Alonso (1996) and Sinev (2009).

Alona guttata Sars, 1862. Littoral species, associated with vegetation. Found in Lake Erhai and in single pond in both winter and spring, in Lake Zibi and smaller reservoirs in spring (loc. W3, W8, W10, W31, S6, S35, S37), never abundant. Species presumed to be cosmopolitan; recent studies of Mexican populations revealed no significant differences from European specimens (Sinev & Silva-Briano, 2012). Common in China (Ji *et al.*, 2015), recorded for Erhai by Lu (1939). For detailed description, see Alonso (1996).

Alona kotovi Sinev, 2012. Rare littoral species, associated with muddy or clay bottom, found in Lake Erhai in winter only (loc. W10, W11, W29). Species described from Vietnam, and recently recorded from Korea (Jeong *et al.*, 2014) and Hainan Island (Sinev *et al.*, 2015). first record of the species for continental China. The only Oriental species of the *quadrangularis*-group (Sinev, 2012, 2016) its area of distribution probably includes India, South-East Asia and South China where it was confused with Palearctic *A. quadrangularis* (O.F. Müller, 1776). Studied specimens (Fig. 1H) share all diagnostic features of *A. kotovi*, including postabdomen with only weakly convex postanal margin (Fig. 1I), and over 12 large setules on anterior margin of thoracic limb I (Fig. 1J) instead of 7-8 in *Alona quadrangularis* s. str. For description, see Sinev (2012).

Anthalona harti harti Van Damme, Sinev & Dumont, 2011. Littoral species, associated with macrophytes. Several specimens in Lake Erhai in winter (loc. W2), among macrophytes. For detailed descriptions see Van Damme *et al.* (2011), for description of Indochina populations see Sinev & Kotov (2012). The first record for Yunnan province. Distributed in tropical Asia, Mediterranean region, and Africa (Van Damme *et al.*, 2011, Sinev & Kotov, 2012). Recorded for China mostly as *Alona verrucosa* Sars, 1901 (Ji *et al.*, 2015).

Camptocercus uncinatus Smirnov, 1971. Littoral species, rather common in Lake Erhai, Lake Xihu and Lake Zibi in both winter and spring; in all lakes among macrophytes and on rocks with filamentous algae. *C. uncinatus* is distributed in southern Europe, Israel, Iraq, Egypt, Ethiopia, Rift Valley of Africa, South-West and East Siberia and Korea (Sinev, 2014). It was recently recorded in Hainan (Sinev *et al.*, 2015). In China, the most frequently reported species of *Camptocercus* is *Camptocercus australis* Sars, 1988 (Ji *et al.*, 2015) but this species is confined to Australia (Sinev, 2015) and all these records most probably belong to *C. uncinatus* as well. Records of subfossil *C. rectirostris* from Erhai (Lu *et al.*, 2016) probably belong to *C. uncinatus*. For description see Sinev (2014), for description of East Asian populations see Kotov *et al.* (2012).

Coronatella rectangula (Sars, 1862) (= *Alona rectangula* Sars, 1862). Littoral species, associated with macrophytes, common in both winter and spring in most water bodies. *C. rectangula* is a common Palaearctic species, distributed from Spain to Far East of Russia, common in China. In East Asia, the species penetrates South up to Peninsular Malaysia and Borneo, but South-East Asian populations slightly differs from Palearctic by narrower postabdomen, probably presenting species-complex (Sinev & Yusoff, 2015; Sinev *et al.*, 2015). Common in China, including Yunnan (Ji *et al.*, 2015), recorded for Lake Erhai (Lu, 1939). For description, see Van Damme & Dumont (2008).

Parthenogenetic females from Dali area (Fig. 2A) have morphology typical for Palearctic populations, with relatively broad postabdomen (Fig. 2B). Inner distal lobe of limb I (Fig. 2C) with rudimentary seta 1 seta 2 curved, claw-like, with two thin spines at the middle and thin posterior portion armed with thin setules, seta 3 thick,

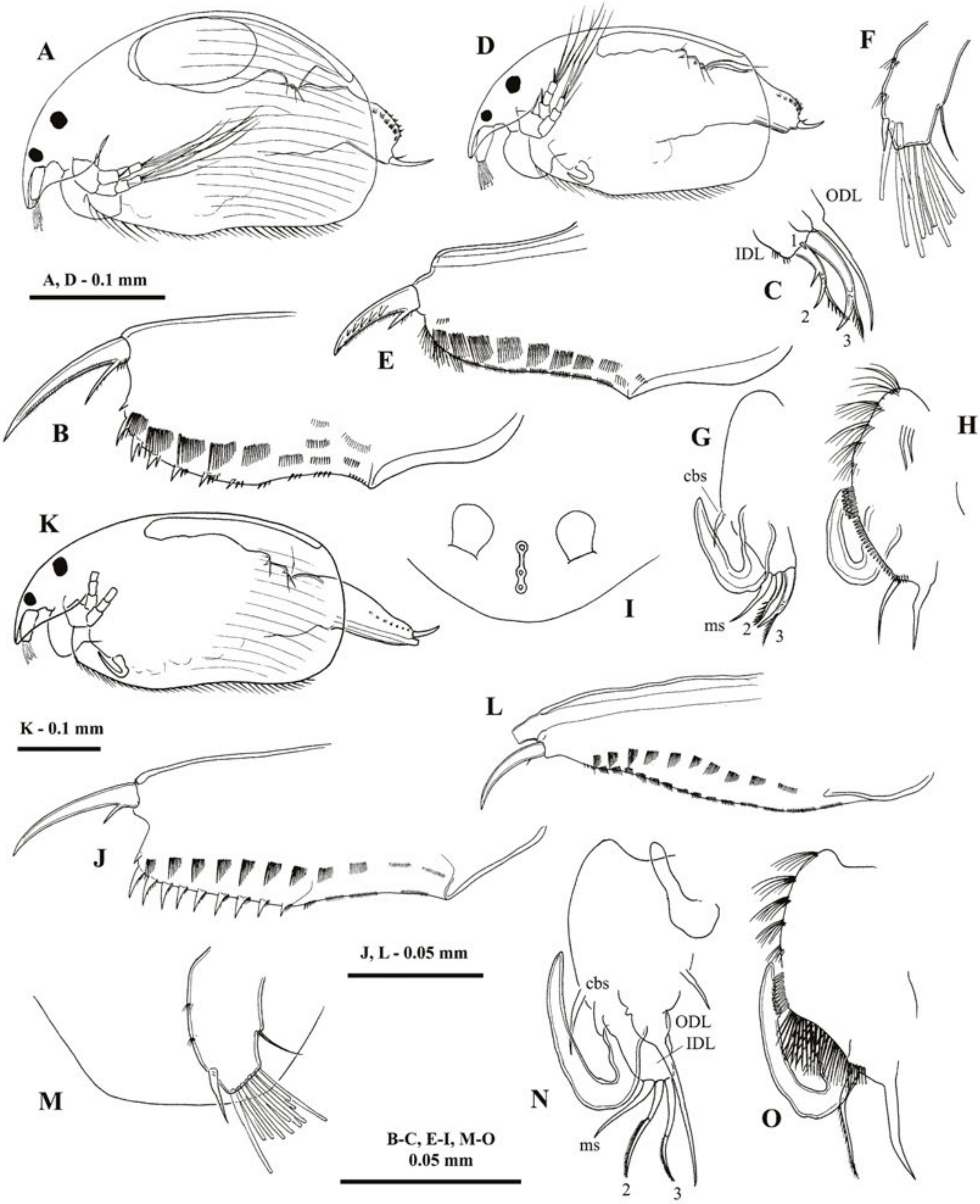

Figure 2. A-H, *Coronatella rectangula* (Sars, 1862) from Lake Erhai (loc. W10). A-C – parthenogenetic female. A, lateral view. B, postabdomen. C, inner distal lobe of thoracic limb I. D-H, adult male. D, lateral view. E, postabdomen. F, antennule. G-H, thoracic limb I (endites not shown). *Flavalona costata* (Sars, 1962) from small reservoir (loc.S14). I-J, parthenogenetic female. I, head pores. J, postabdomen. K-O, adult male. K, lateral view. L, postabdomen. M, antennule and outline of rostrum. N-O, thoracic limb I (endites not shown). Abbreviations: cbs – copulatory brush seta; IDL – inner distal lobe; ms – male seta; ODL – outer distal lobe. Coronatella rectangula *(Sars, 1862) del lago Erhai (loc. W10). A-C – hembra partenogenética. A, vista lateral. B, postabdomen. C, lóbulo distal interior del limbo torácico I. D-H, macho adulto. D, vista lateral. E, postabdomen. F, anténula. G-H, limbo torácico I (no se muestran los endites).* Flavalona costata *(Sars, 1962) de un pequeño embalse (loc.S14). I-J, hembra partenogenética. I, Poros de la cabeza. J, postabdomen. K-O, macho adulto. K, vista lateral. L, postabdomen. M, anténula y silueta del rostrum. N-O, limbo torácico I (no se muestran los endites). Abreviaciones: cbs – seta pincelada copulatoria; IDL – lóbulo interior distal; ms – seta de macho; ODL – lóbulo distal exterior.*

curved, claw-like, with single thick spine at the middle and thin posterior portion armed with setule; seta 2 shorter and two times thinner than seta 3, seta 3 slightly longer than outer distal lobe seta. Morphology of males from East Asian population were never studied in detail, so full description is provided.

Male. Body (Fig. 2D) low oval, height-length ratio about 0.6. Ocellus and eye are of the similar size as in female, eye two times larger than the ocellus.

Postabdomen (Fig. 2E) is short, of moderate width, with subrectangular distal portion. Ventral margin almost straight. Gonopores located at the end of postabdomen. Distal margin almost straight, distal angle broadly rounded. Postanal and anal margin almost straight. Distal part of postabdomen 1.5 times longer than preanal part; anal and postanal portion of similar length. Postanal angle not defined, preanal angle well-defined, prominent. Clusters of short setules in place of marginal denticles, lateral fascicles of setules similar to those of female, but distal setule in each fascicle thinner, of similar thickness as the others. Postabdominal claw short, about 2/3 length of postanal margin, weakly curved, with short thick basal spine about 1/4 length of claw itself. There are 4-5 thick short spines on inner side of claw, these spines are not present in other species of *Coronatella*.

Antennule (Fig. 2F) moderately long, length about 2.5 widths. Male seta short, arising at 3/4 distance from the base and reaching to the end of antennule. Two lateral and ten terminal aesthetascs, longest of them slightly shorter than antennule itself. All aesthetascs projecting beyond anterior margin of the head shield. Thoracic limb I (Fig. 2G-H) with short U-shaped copulatory hook two times shorter than limb itself. Copulatory brush present; copulatory brush seta short. Ventral face of limb below them with about 20 short thick setules. Inner distal lobe without seta 1; setae 2 and 3 much shorter and thinner than in female; seta 2 slender, without any spines at the middle, distal portion armed with thin setules; seta 3 moderately thick, curved, with single spine at the middle and thin posterior portion armed with thin setules, longer than seta 2; male seta curved, shorter than seta 3.

Morphology of males fully agrees with earlier descriptions (Frey 1988a; Alonso, 1996; Van Damme & Dumont 2008) with one exception – lateral aesthetascs on antennulae were never reported for males of *C. rectangula*. Due to small size of the animal it is unclear if they were overlooked in previous studies or are present only in studied population.

Flavalona costata (Sars, 1962) (former *Alona costata* Sars, 1862). Littoral species, associated with macrophytes, common in both winter and spring in most water bodies. Palearctic species, in East Asia penetrating South to the Hainan Island and North-East Thailand (Sinev, 2016). Common in China, including Yunnan (Ji *et al.*, 2015), recorded for Lake Erhai by Lu (1939). Studied females have typical for the species morphology of postabdomen (Fig. 2J) and head pores (Fig 2I), with length of lateral head pores about half distance between anterior and posterior main head pores, and with rounded lateral head pore pockets. Ephippial females were found in Lake Erhai in winter (loc. W10) and in small reservoir and fish pond in spring (loc S.14, S15). Males of *F. costata* are rarely recorded for East Asian populations, so their full description is provided.

Body (Fig. 2K) low oval, dorsal margin only weakly convex. Maximum height at the middle of the body, height/length ratio c. 0.55. Ocellus smaller than eye. Rostrum short, slightly truncated (Fig. 2M).

Postabdomen (Fig. 2L) moderately long, narrowing distally in postanal part. Ventral margin wavy in studied specimens. Basis of claws bordered from distal margin by clear incision. Genital process short, about 1/4-1/3 length of postabdominal claw. Distal margin very short, distal angle obtuse. Dorsal margin convex in anal portion, straight in postanal portion. Preanal and postanal angles not defined. Clusters of short setules in place of marginal denticles, distalmost cluster consisting of 3-5 thick short setules. Lateral fascicles as in female. Postabdominal claw slightly longer than preanal portion of postabdomen, curved. Basal spine short and thin, visible only under oil immersion.

Antennule (Fig. 2M) with 12 terminal aesthetascs, longest about 3/4 length of antennule. Male seta arising at 1/4 length from tip, about 1/3 length of antennule. Thoracic limb I (Fig. 2N-O) with U-shaped copulatory hook, two times shorter than limb itself. IDL with three setae. IDL setae 2 and 3 thin, of similar length. Male seta curved, shorter than seta 2. Copulatory brush seta about 2/3 length of IDL seta 2. Ventral face of the limb under the copulatory brush with row of about 20 long stiff setules.

Morphology of males fully agree with the previous descriptions of European populations of the species (Alonso, 1996; Sinev, 1999; Hudec, 2010; Sinev & Dumont, 2016). For detailed description see Sinev & Dumont (2016).

Graptoleberis testudinaria (Fischer,1851). Littoral species, associated with macrophytes with submerged leaves. Uncommon in the area, found in Erhai, Xi Hu and Zibi, and in some ponds, more frequent in spring. Species recorded worldwide, but probably composes a species-complex, common in China (Ji *et al.*, 2015), recorded for Lake Erhai by Lu (1939). For description of European populations, see Alonso (1996), Hudec (2010).

Leydigia ciliata Gauthier, 1939. Benthic species, associated with muddy or clay bottom. Found in Erhai and Chianghai lakes in spring only. Known in Africa, tropical Asia, common in Oriental region (Kotov, 2009). Common in China (Ji et al., 2015). For detailed description, see Kotov *et al.* (2003).

Oxyurella tenuicaudis (Sars, 1862). Littoral species, associated with vegetation. Rare in the area; few specimens in Lake Erhai in winter (loc. W6). Widely distributed Palearctic species; frequently recorded in China, including Yunnan; southmost records in Guangdong province (Ji *et al.*, 2015), but, according to these authors, some records, especially from South China, can be erroneous. Not found in Hainan Island (Sinev *et al.*, 2015), where it is substituted by tropical species *Oxyurella singelansis*. For description, see Alonso (1996) and Hudec (2010).

Family Chydoridae, subfamily Chydorinae

Alonella excisa (Fisher, 1854). Littoral species, associated with vegetation. Rare in the area, several specimens found in Lake Erhai once in winter (W6) and once in spring (loc. S32), and in Lake Jainhu in winter (loc. W25). Common in China, including Yunnan (Ji *et al.*, 2015), recorded for Lake Erhai by Lu (1939). The species presumed to be cosmopolite (Smirnov, 1996). For description, see Alonso (1996).

Chydorus sphaericus (O.F. Müller, 1776) s. lato. Eurybiotic littoral species, sometimes encountered in pelagic zone. The most common species in the area, found in all types of water bodies in littoral samples. *Chydorus sphaericus* s. lato in Eurasia is a complex of species with identical morphology of parthenogenetic females (Belyaeva & Taylor, 2009; Klimovsky & Kotov, 2015; Kotov *et al.*, 2016). *Chydorus sphaericus* s. lato is common in China, including Yunnan (Ji *et al.*, 2015), recorded for Lake Erhai by Lu (1939). Two ephippial females were found in Lake Erhai in winter (loc. W11). These specimens had ephippia with single egg, typical for most species of *C. sphaericus* group, while two species of the group found in North-East Palearctic, *C. belyaevi* Klimovsky & Kotov, 2015 and *C.* cf. *biovatus* Frey, 1985 have ephippia with two eggs (Klimovsky & Kotov, 2015).

Disparalona (Disparalona) ikarus Kotov & Sinev, 2011. Species associated with open littoral. Rare, in spring in Lake Erhai (loc. S40) and two small reservoirs (loc. S14, S25). Species so far is known from Amur river basin in Russia, South Korea and Hainan Island (Kotov & Sinev, 2011; Kotov *et al.*, 2012; Sinev *et al.*, 2015). It is the first record of the species in continental China. For description, see Kotov & Sinev (2011) and Kotov *et al.* (2012).

Disparalona (Mixopleuroxus) chappuisi Brehm, 1934. Littoral substrate-associated species. Common, encountered in most water bodies in both spring and winter, more abundant among macrophytes. Species known from Africa and tropical Asia, in East Asia penetrating north to Far East of Russia (Neretina et al., 2018). Population of *D. chappuisi* from East and South-East Asia, including these from China (see Ji *et al.*, 2015; Sinev *et al.*, 2015) were recorded as *D. hamata* Birge,1879, but recent revision clarified its status (Neretina *et al.*, 2018). For detailed description see Neretina *et al.* (2018).

Pleuroxus (Pleuroxus) aduncus (Jurine, 1820). Littoral species, associated with vegetation. Uncommon, found in vegetated areas of Lake Erhai in both winter (loc. W12, W28) and spring (loc. S29, S32, S33), in spring also found in Lake Xihu (loc. S3) and fish ponds (loc. W15). The species was considered cosmopolitan (Smirnov, 1996), but its absence in South Hemisphere was proved recently (Smirnov *et al.*, 2006). Common in China, but some records, especially from South part, can be unreliable (Ji *et al.*, 2015); recorded for Erhai by Lu (1939). Dali area is a one of Southmost sure records for the species. For description, see Smirnov (1996) and Frey (1991).

Pleuroxus (Picripleuroxus) laevis Sars, 1862. Rare littoral species, associated with vegetation, found in winter in five locations in Lake Erhai only (loc. W1, W7, W9, W13, W29), absent in spring. Palearctic species (Smirnov, 1996). Common in China, including Yunnan (Ji *et al.*, 2015). The first record for Dali Area. For description see Smirnov (1996) and Frey (1988b).

Pleuroxus (Picripleuroxus) quasidenticulatus (Smirnov, 1996). Littoral substrate-associated species. Most common species of *Pleuroxus* here, found mostly among macrophytes, but also on open littoral; in Erhai, Xi Hu, Zibi and Chianghai lakes and in various ponds, common and abundant in spring, infrequent in winter. recorded frpm Australia, Iran, South-East Asia, China, Korea and Far East of Russia (Smirnov, 1996; Sinev & Korovchinsky, 2013; Sinev & Sanoamuang, 2013; Kotov *et al.*, 2017). For description, see Sinev & Sanaomuang (2013).

Pseudochydorus bopingi Sinev, Garibian & Gu, 2016. Littoral species, feeding on the bodies of dead microcrustaceans. Found in limited numbers (several specimens per sample) in samples with abundant littoral cladocera, in Lake Erhai in both winter and spring, and in Zibi, Chianghai and two smaller reservoirs (loc. S14, S25) in spring. The species was recorded in South Vietnam, Hainan Island, Hunan and Hubei provinces of China (Sinev *et al.*, 2016). The first record for Yunnan. Earlier *Pseudochydorus* records from China were attributed to *P. globosus* (*et al.*, 2015), but all populations from South-East China probably belong to *P. bopingi*. For description, see Sinev *et al.* (2016).

Family Bosminidae

Bosmina (Bosmina) longirostris (O. F. Müller, 1776). Pelagic plankton species. Common in the area, found in most large water bodies in the area and in some ponds, more frequent in spring. Recorded worldwide, according to Chatterjee *et al.* (2013), who refer to unpublished genetic data of Taylor and Kotov, one of the few truly cosmopolitan cladocera. Common in China, including Yunnan (Xiang *et al.*, 2015). For description see Alonso (1996).

DISCUSSION

Among the 34 species found in the area, 31 were present in Lake Erhai and 8 of were found only in Lake Erhai (see Table 1). In our opinion, this reflects a higher diversity of biotopes in the lake, for example, clusters of water caltrop and open rocky littoral exists there, while these are not present in other lakes. Number of species in two intensively sampled lakes, Xihu and Zibi, was low, only 14 species in each. Three other large lakes, Jianhu, Chenghai, Shuizhangdi, were sampled less intensively, and their diversity probably underestimated. Only 13 species were found in the ponds, and only 11 in various reservoirs with severe water fluctuations.

Lake Erhai region winter-spring fauna is conspicuous by the absence of several common groups of Cladocera, including the genera *Diaphanosoma* and *Moina*, both quite common in Central Yunnan, including Kunming area. Kunming area was sampled by the authors at the same time as Dali Area, and at least two species of *Moina* and three species of *Diaphanosoma* were present in the area, being dominating in many samples. The only species of *Diaphanosoma* recorded in Dali Area is *Diaphanosoma brachyurum*, present in Lake Erhai pelagic zone in Jule-November (Yang et al., 2014). *Diaphanosoma dubium* and *Moina micrura* were found in the area to be usual by the second author (Gu, unpublished). Several tropical species, reported for ponds and paddy fields of Dali area (see Xiang *et al.*, 2015); *Ephemeroporus barroisi*, *Leberis diaphanus*, *Dunhevedia crassa* King, 1853, were not found during our study neither. With these records, the number of taxa in Dali area could be increased until 40 species.

Lake Erhai region fauna during winter-spring is composed by three ecological groups of species. Most cladocera here (25 species), including all plankton taxa, are either Palearctic or widely distributed taxa. For some of them (*Ceriodaphnia dubia*, *Ceriodaphnia reticulata*, *Daphnia galeata*, *Daphnia pulex*, *Pleuroxus aduncus*, *Oxyurellla tenuicaudis*, *Ilyocryptus cuneatus*), Dali Area is on the southmost margin of distribution area. For these taxa, Dali Area is an "area of penetration" sensu Korovchinsky (2004, 2006), since their "areas of primary distribution" are in North Palearctic. Their appearance in Dali Area is probably a reflection of specific local conditions (moderately high altitude).

The second group are East Asian taxa (four species, *Sida ortiva*, *Disparalona ikarus*, *Pseudochydorus bopingi* and *Alona kotovi*) distributed from South-East Asia to Amur basin (Kotov, 2016). The third group are predominantly Paleotropical taxa (five species, *Scapholeberis kingi*, *Macrothrix spinosa*, *Anthalona harti*, *Leydigia ciliata* and *Pleuroxus quasidenticulatus*), they are rare in the area in spring-winter, only the latter species became frequent in spring. All these species have areas of primary distribution in the Oriental region and South China, but penetrate further north in lowland East China (see Korovchinsky, 2013).

In both sampling seasons, only parthenogenetic females were found in a majority of species. Few gamogenetic females and males were found in few common and abundant species: in winter for *Coronatella rectangula*, *Chydorus spahaericus*, *Flavalona costata* and *Daphnia galeata*, and in spring for *Flavalona costata* only. This shows that Dali area climate is mild enough to allow Palearctic species to overwinter as active populations, giving them an advantage over tropical species, which should overwinter at resting egg stage.

Many Cladocera described from Europe historically were presumed to be distributed across the whole Palearctic region. However, recent studies revealed that such taxa are often composed of sibling species, usually with limited ranges. For example, investigation of *Chydorus sphaericus* complex (Belyaeva & Taylor, 2009; Klimovsky & Kotov, 2015; Kotov *et al.*, 2016) revealed that *C. sphaericus* s. str. is not present in the East Palearctic, being substituted there by *C.* cf. *biovatus* Frey, 1988, *C. belyaevae* Klimovsky & Kotov, 2015 and several not yet described species of the clade. Genus *Leptodora*, presumed to be monotypical, is composed of at least two species distributed in West and East Palearctic (Korovchinsky, 2009); similar situation was revealed for genus *Polyphemus* (Xu *et al.*, 2009). *Pleuroxus trigonellus* is substituted by sibling species in East Palearctic (Garibian *et al.*, 2018). On the other hand, *Daphnia curvirostris* is distributed throughout the whole Palearctic region, but in East Asia it coexists with a cluster of local sibling-species (Kotov *et al.*, 2006; Ishida *et al.*, 2006).

Our data on two species of Aloninae, *Flavalona costata* and *Coronatella rectangula*, show that these populations are similar to European ones in both female and, more importantly, male morphology. In the family Chydoridae, differences in male morphology between sibling species are usually more pronounced than those in females (Frey, 1988b; Sinev, 1999, 2018; Neretina *et al.*, 2018). Some sibling species can be morphologically distinguished by male morphology only (Klimovsky & Kotov, 2015; Sinev & Atroschenko, 2011; Sinev, 2013; Garibian *et al.*, 2018). Our data suggests that *F. costata* and *C. rectangula* are monotypic within whole Palearctic region, and patterns of East-West distribution in Palearctic cladocera are rather variable even within one family.

All four species of the Cladocera recorded for continental China for the first time are substrate-associated. Two of these, *Alona kotovi* and *Camptocercus uncinatus* were previously recorded for Yunnan as *A. quadrangularis* and *C. australis* or *C. rectirostris*, respectively (Ji *et al.*, 2015); these taxa are not present in South-East China. Our data confirms persistent problems with identifications of substrate-associated Cladocera in China (Xiang *et al.*, 2015; Ji *et al.*, 2015).

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Zooplankton changes at six reservoirs in the Ebro watershed, Spain

Muñoz-Colmenares, M. E.[1,*], E. Vicente[1,2], J. M. Soria[1,2] and M. R. Miracle[2,†]

[1] Programa de Doctorado en Contaminación, Toxicología y Sanidad Ambientales, Departament de Microbiologia i Ecologia, Universidad de Valencia, E-46100 Burjassot, Spain.
[2] Institut Cavanilles de Biodiversitat i Biologia Evolutiva, Universitat de València, 46980 - Paterna, Spain.

[*] Corresponding author: mamucol@alumni.uv.es

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ABSTRACT

Zooplankton changes at six reservoirs in the Ebro watershed, Spain

In the present study, six reservoirs of the Ebro watershed were sampled during summer and autumn of 2016, with the objective of recognizing the zooplankton community, the environmental variables that are correlated with them and update the species checklist. We identify 40 zooplankton species among reservoirs: 21 rotifer species, 10 cladocerans, 8 copepods, and the veliger larvae of the invasive zebra mussel. Species that had higher abundances and biomass were: the rotifer *Polyarthra dolichoptera* (up to 278 ind/L), the cladoceran genera *Daphnia* spp. and *Ceriodaphnia* spp., the copepods *Copidodiaptomus numidicus* (83 ind/L), *Acanthocyclops americanus* (72 ind/L), *Eudiaptomus vulgaris* (62 ind/L) and zebra mussel *Dreissena polymorpha* (540 ind/L). In general, the smaller species were dominant during the summer while the larger species were dominant in the autumn. The density and biomass of zooplankton in four out of six reservoirs during the summer were double that in autumn. The main physicochemical variables correlated with the zooplankton species through a Canonical Correspondences Analysis (CCA) were: chlorophyll a, Secchi disk, total phosphorus, pH and depth. We report for the first time the species presented at the Ullibarri-Gamboa reservoir, and present new species registered in the reservoirs compared with those reported in previous studies. For the first time, the presence of the zebra mussel was detected at La Sotonera reservoir. This indicate its expansion throughout the watershed, suggesting that knowledge of zooplankton species and the changes that occur through time can be a tool for reservoirs and watershed management.

Key words: zooplankton community, reservoirs, Ebro watershed, physicochemical parameters, species seasonal variation

RESUMEN

Cambios en el zooplancton en seis embalses en la cuenca del Ebro, España

En el presente estudio, seis embalses de la cuenca del Ebro fueron muestreados durante las estaciones de verano y otoño de 2016, con los objetivos de conocer la estructura de la comunidad de zooplancton, las variables ambientales con las que se correlación y actualizar los listados de especies. Se identificaron 40 especies de zooplancton entre todos los embalses; 21 especies de rotíferos, 10 de cladóceros, 8 de copépodos y las larvas veligeras del invasivo mejillón cebra. Algunas de las especies que tuvieron mayores abundancias y biomasa fueron: el rotífero Polyarthra dolichoptera *(hasta 278 ind/L), los géneros de cladóceros* Daphnia *spp, y* Ceriodaphnia *spp., los copépodos* Copidodiaptomus numidicus *(83 ind/L),* Acanthocyclops americanus *(72 ind/L),* Eudiaptomus vulgaris *(62 ind/L) y el mejillón cebra* Dreissena polymorpha *(540 ind/L). En general, durante el verano las especies de menor tamaño fueron las dominantes mientras que en el otoño tuvieron una mayor dominancia las especies de mayor tamaño. La densidad y biomasas del zooplancton en cuatro de los seis embalses durante el verano fue el doble que en otoño. Las principales variables fisicoquímicas correlacionadas a las especies de zooplancton a través de un Análisis de Correspondencias Canónicas (ACC) fueron: clorofila a, disco de Secchi, fosforo total, pH y la profundidad. Se reportan por primera vez las especies presentes en el embalse de Ullibarri-Gamboa, además de que se presentan nuevas especies registradas en el resto de los embalses contra aquellas reportadas en estudios anteriores. Se detecto por primera vez la presencia del mejillón cebra en el embalse de La Sotonera, indicando su expansión a través de la cuenca, esto nos sugiere que el conocimiento de las especies del zooplancton y los cambios que presentan a través del tiempo puede ser una herramienta para el manejo de los embalses y la cuenca.*

Palabras clave: *comunidad del zooplancton, embalses, cuenca del Ebro, parámetros fisicoquímicos, variación estacional de especies*

INTRODUCTION

Zooplankton is, an important component inside the freshwater ecosystem, playings a big role in the transfer of energy in the aquatic food web between primary producers and higher consumers, while significantly contributing to the recycling of nutrients (Lampert & Sommer, 1997).

Besides their essential role in trophic levels of aquatic environments, this group can also provide valuable information that other groups cannot. For example, changes in certain metrics such as, size, proportion of large and small zooplankton, mean of body weight and proportion of resting eggs together with the zooplankton:phytoplankton biomass ratio, which can indicate a "top-down" process (Jeppesen *et al.*, 2011). Top-down control is one of the main attributes of zooplankton. This occurs when zooplankton consumes high quantities of phytoplankton and becomes a pressure factor, this pressure can determine the composition of phytoplankton assemblage and decrease their abundances and biomass (Naselli-Flores & Rossetti, 2010). On the other hand, the zooplankton community can respond quickly to phytoplankton blooms during the bottom-up control (Carpenter *et al.*, 1985), such as, changes in the biomass, the proportion of calanoids copepods and numbers of rotifers could indicate this process (Jeppesen *et al.*, 2011). Due to their pivotal position in the transfer of nutrients and energy in aquatic food webs and the valuable data they can provide, it is essential to have a wide knowledge of zooplankton composition and the factors related to this group (Caroni & Irvine, 2010).

All the species and individuals that make up the zooplankton community exhibit diverse responses to changes (Stemberger *et al.*, 2001). These changes can be done by biotic (e.g. food availability, predation and competition) and abiotic (physical and chemical habitat conditions: temperature, dissolved oxygen, pH, etc.) factors, as both can affect the species richness, increasing or decreasing their abundances and biomass, and promoting shifts in their diversity (Jeppesen *et al.*, 2000; Wetzel, 2001; Dodson *et al.*, 2009; Bonecker *et al.*, 2013). Hence, studies focused on such factors can provide useful information to manage natural resources (Gulati *et al.*, 1990) as well as the understanding of how its community structure (species richness, density and abundances) varies with time and in different aquatic systems (Dodson *et al.*, 2009; Boix *et al.*, 2008).

Seasonal variation also has an important role in waterbodies. Over the course of a year many environmental variables can suffer big changes depending on the season (Margalef, 1983). On a regional and local scale, these seasonal changes in natural components, in addition to anthropogenic pollution, can impact on aquatic communities and affect the zooplankton groups in different ways (Tavernini *et al.*, 2009).

Many studies have correlated density, species richness and the presence or absence of zooplankton, for example, rotifers (Sladečeck, 1983; Ejsmont-Karabin, 1995, 2012; May & O'Hare, 2005) and micro-crustaceans (Pinto-Coelho *et al.*, 2005) to the trophic gradient. Moreover, zooplankton, can be an element in evaluating the trophic state of reservoirs and lakes (Haberman *et al.*, 2007; Haberman & Haldna, 2014) and a good indicator of the different trophic states related to natural processes, man-made activities and climate changes (Jeppesen *et al.*, 2011). Recently, in man-made reservoirs have proved that even zooplankton density can be a tool to determinate the trophic state of a large watershed in Spain (Garcia-Chicote *et al.*, 2018). Although the Water Framework Directive has the aim of evaluating the European waters through several Biological Quality Indicators, zooplankton and its valuable data is not included as one of these indicators.

Despite the Ebro watershed being the second large watershed in Spain, studies related to zooplankton presence in the reservoirs are few and focus principally on rotifera phylum´s description or distribution (De Manuel & Armengol, 1993; De Manuel, 2000). In the present study we focus on zooplankton communities of six reservoirs located across the watershed. These were chosen due to the fact that existing data is more than 30 years old (De Manuel & Jaumel, 1993) or no previous data was available. The lack of information of these reservoirs throughout the last three decades could significate changes in species composition and non-detected invaders. Therefore, it´s important to update the current knowledge on zooplanktonic fauna in this watershed and know how environmental variables can affect the composition of communities through seasons. Thus, all this information could be a helpful tool for reservoirs management.

The main objectives of this study were; first, report and compare the zooplankton composition (species richness, density and biomass) during two studied seasons (summer and autumn) in six reservoirs at the Ebro watershed. Second, determine the environmental variables related to the zooplankton groups structure (density and biomass). Third, update information on the zooplanktonic fauna and verify if new species are present in the reservoirs compared with available data of previous studies.

MATERIAL AND METHODS

Study area

The data presented in this study was obtained from six reservoirs, located in different areas and altitudes along the Ebro watershed (Fig. 1). Each reservoir was sampled at the beginning of two different seasons in 2016: summer (last week of June) and autumn (last week of September). One sampling point was established at each reservoir in the deepest part of the reservoir at 300-500 meters from the dam.

Environmental Variables

For each reservoir the following variables were measured along the water vertical profile, temperature, conductivity, dissolved oxygen, pH, turbidity and chlorophyll *a*, all *in situ* measurements, by

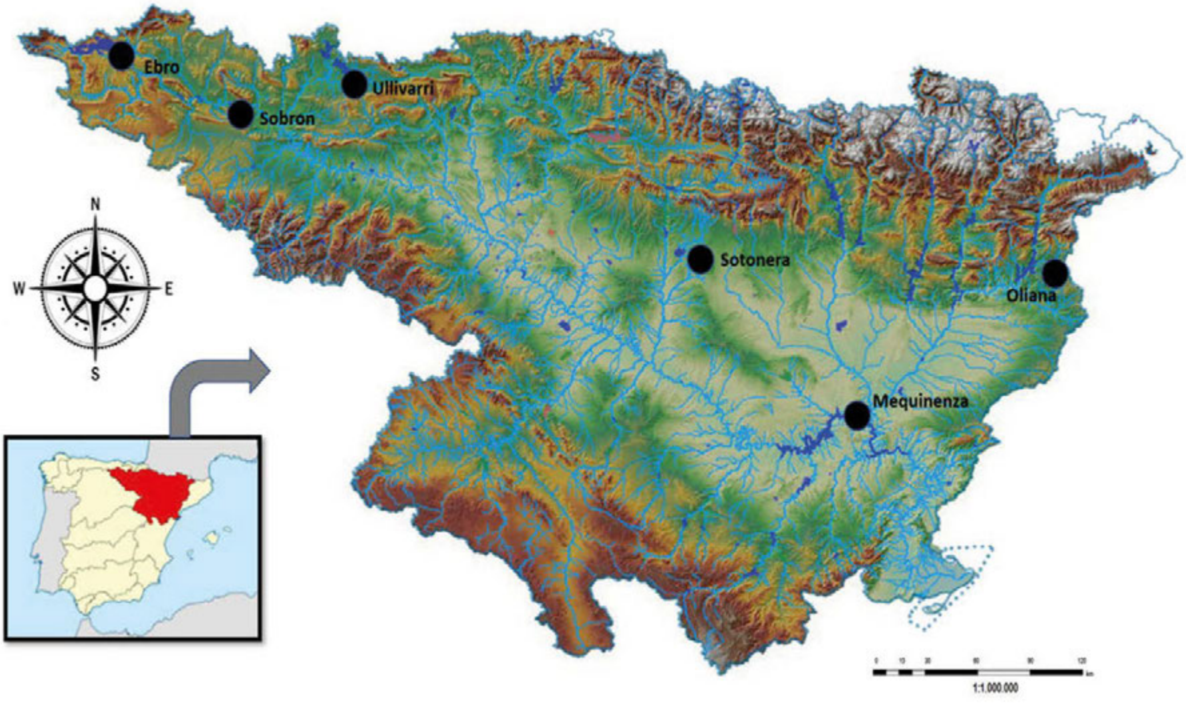

Figure 1. Location of the Ebro Watershed with the sampled reservoirs. *Localización de la Cuenca del Ebro con los embalses muestreados.*

means of a multiparametric devise Sea-Bird 19 plus V2. The depth of the photic zone was calculated by measuring the light penetration using a quantometer. The water transparency was determined measuring the Secchi disk depth (SD). An integrative water sample was collected from the photic zone of each reservoir using a 25 mm ballasted tube technique for *ex-situ* analyses (Vicente *et al.*, 2005). For measurements of the following variables, we used the standard methodology (APHA, 1998) described for suspended solids (APHA 2540D), turbidity (ISO7027-1999), total nitrogen (TN) (APHA method 4500-N C), total phosphorus (TP) (4500-P B/APHA 4500-P C), and chlorophyll *a* (Shoaf & Lium, 1976). The complete data set of environmental variables can be found at C.H.E. (2016).

To estimate the reservoir´s trophic conditions, we used the criteria of the trophic state index (TSI) (Carlson, 1977). The TSI´ values of each reservoir were obtained with the following formulae (Carlson & Simpson, 1996):

Total phosphorus; $\mathrm{TSI\,(TP)} = 14.42 \ln (\mathrm{TP}) + 4.15$
Chlorophyll *a*, $\mathrm{TSI\,(Chl\text{-}}a) = 9.81 \ln (\mathrm{Chl\text{-}}a) + 30.6$
Secchi disk, $\mathrm{TSI\,(SD)} = 60 - 14.41 \ln (\mathrm{SD})$

Total phosphorus and Chl-*a* are measured in micrograms per liter (µg/L) and Secchi disk depth is expressed in meters. TSI is the average value of the three above mentioned variables.

$$\mathrm{TSI}' = [(\mathrm{TSI(TP)} + \mathrm{TSI\,(Chl\text{-}}a) + \mathrm{TSI\,(SD)})/3]$$

Zooplankton samples

The zooplankton samples were collected using a Ruttner bottle with a capacity of 2.7 L. For each reservoir were taken two Ruttner bottles to obtain 5.4 liters of water sample, then the sample was filtered through 30 µm mesh size Nytal, fixed with formaldehyde at 4 % final concentration and stored in a hermetic glass vial. The sample depth was established in each reservoir at the beginning of oxygen decline, where has been reported as the richest zone of zooplankton fauna (Miracle & Vicente, 1983). Also, a zooplankton vertical tow net of 50 µm mesh size Nytal was towed from 30 m deep to the surface, collected and fixed with formalin. These vertical tow net samples were taken mainly for taxonomic purposes.

Zooplankton species were identified using the following guides: Ruttner-Kolisko (1974), Koste (1978), Nogrady *et al.*, (1995) and Nogrady & Segers (2002) for rotifers, Alonso (1996) for cladocerans, and Dussart (1967, 1969) for copepods.

For quantitative results, we used the samples taken from the Ruttner bottles, all individuals were counted using a Sedgewick Rafter-type counting chamber under inverted microscopy. After individuals were counted and densities were obtained, we calculate the biomass, to determine it, a minimum of 30 specimens of all species were measured and using the formulas that relate the total length with the dry weight of the specimens were obtained the corresponding conversion factors (Ruttner-Kolisko, 1977; Dumont *et al.*, 1975; Culver *et al.*, 1985). The Shannon–Wiener diversity index (H’) (Shannon & Weaver, 1963) was calculated from data on the abundance of zooplankton for each reservoir at both seasons.

Statistical analysis

The correlation coefficients between zooplankton data and the environmental factors were calculated by linear Pearson correlations. Analysis of similarity (ANOSIM) tests were performed on the zooplankton data to determine which, if any, reservoirs showed significant differences in zooplankton community structure between the two seasons. ANOSIM is a nonparametric analogue to analysis of variance and tests for multivariate differences between groups based on Bray-Curtis distance and rank dissimilarity. Also, we ran a similarity percentage routine (SIMPER), to test which zooplankton species were contributing to the community changes. The SIMPER routine uses average Bray-Curtis dissimilarities between all pairs of sites to produce a percent contribution from each species, identifying the species most responsible for the dissimilarity (Clarke & Warwick, 2001).

To determine the influence of different factors on zooplankton we performed two canonical correspondence analysis (CCA). For the first, we

Table 1. Complete data of physicochemical variable measurements during both seasons, data modified from C.H.E (2016). *Datos completos de los parámetros fisicoquímicos medidos durante las dos sesiones. Datos modificados de C.H.E. (2016).*

		Summer						**Autumn**					
Parameter	**unit**	ULL	MEQ	EBR	OLI	SOB	SOT	ULL	MEQ	EBR	OLI	SOB	SOT
Temperature	**°C**	20.80	23.62	18.15	18.87	21.59	23.50	19.49	24.64	18.41	22.99	17.77	23.29
Disolved oxygen	**mg/L**	7.16	6.79	7.84	8.70	7.31	8.04	5.85	3.52	7.95	7.45	6.87	7.29
Conductivity	**µS/cm**	244	815	188	187	324	331	219	1288	195	270	255	318
pH		8.43	8.24	8.18	8.45	8.31	8.34	8.09	7.94	7.87	8.35	7.89	8.38
Depth	**m**	23	57	20	50	27	20	21	53	14	37	27	12
Secchi	**m**	3.50	3.70	4.50	2.90	1.70	2.25	7.75	3.80	1.40	2.70	2.00	1.10
Suspended solids	**mg/L**	1.12	2.13	1.29	4.32	4.47	3.40	0.79	2.02	7.41	4.11	3.39	7.56
Turbidity	**NTU**	1.88	1.88	2.89	3.80	4.37	1.61	1.55	1.22	6.99	3.86	4.47	4.74
Alkalinity	**Meq/L**	2.28	3.12	1.24	1.52	2.32	2.52	2.00	2.85	1.24	1.92	1.88	2.24
Chl-*a*	**µg/L**	2.26	3.66	2.26	6.73	11.13	3.38	1.37	3.69	5.00	21.14	3.03	3.69
TN	**µg/L**	660	217	450	680	810	450	324	1692	427	622	484	276
TP	**µg/L**	9.41	9.12	13.54	80.78	25.50	8.39	22.44	13.00	26.48	25.00	19.29	16.00

analyzed those variables that are corresponded to the principal zooplankton species. For the second, we performed an analysis using the zooplankton groups (rotifers, copepods, cladocerans and zebra mussels). For each CCA we included the densities and or biomass of zooplankton and the following environmental variables (temperature, dissolved oxygen, conductivity, turbidity, pH, Secchi disk, depth, nutrients (TP and TN) and Chl-*a*. In order to normalize the data, they were transformed logarithmically Log(x + 1), except for pH. The models were tested using Monte Carlo permutation (n = 499). Nauplius, copepodites and bdelloid rotifers were excluded since they were not identified to species level. Both CCAs were executed using the Canoco 4.5 for Windows computer program (Ter Braak & Šmilauer, 2002).

RESULTS

Environmental parameters

During the two seasons of this study the physical and chemical parameters varied at the different reservoirs, complete data is reported in Table 1. The water temperature on average was higher during summer in all waterbodies, except at Oliana, where it was higher in autumn. In general, the dissolved oxygen presents higher values during summer than those in autumn. The pH values do not show an important difference between seasons because the buffer effect of the bicarbonate in the waters and the conductivity values were stable (with exception of Mequinenza during autumn with a peak of 1288 µS/cm). Suspended solids in both seasons were similar in four of the six reservoirs, however, data from the Ebro and La Sotonera reservoirs during the autumn were double compared to the summer data. The Secchi disk visibility presented a wide variability among reservoirs and seasons: Ebro, Oliana and La Sotonera had higher values in summer, nevertheless, Ullibarri-Gamboa, Mequinenza and Sobrón were higher during autumn.

In the case of Chl-*a*, higher values were presented during the summer at Ullibarri-Gamboa and Sobrón, and during the autumn at Ebro and Oliana. The Oliana reservoir (autumn) had the biggest Chl-*a* concentration of all the study (21.14 µg/L). Finally, Mequinenza and La Sotonera had similar values during both seasons (average of 3.3 µg/L and 3.5 µg/L respectively). Total Nitrogen (TN) values at 5 of the reservoirs were higher during summer, only Mequinenza presented a high peak in autumn (1692 µg/L). The higher values of total phosphorus (TP) were reported in autumn, except at Oliana (80.78 µg/L) and Sobrón (25.5 µg/L), where the higher values were during summer.

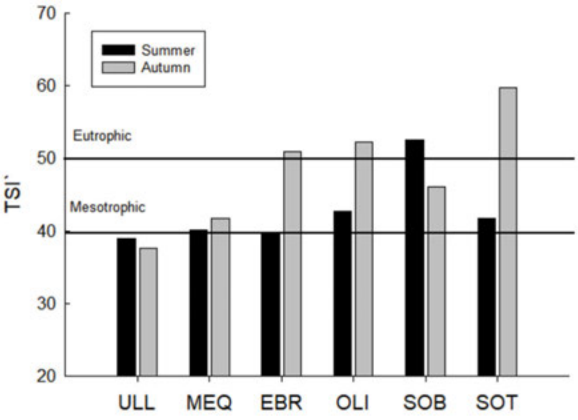

Figure 2. Reservoir TSI´ values, black charts (summer) and gray charts (autumn). *Valores de TSI´ de los embalses, barras en negro (verano) y gris (otoño).*

Trophic state

With the data obtained, we calculated the Trophic State Index (TSI) for every reservoir and season, the waterbodies were ordinated according to their TSI from lower to higher values. Ullibarri-Gamboa shows the lower trophic value in both seasons (39.01, summer and 37.70, autumn) and is classifiedd as oligotrophic. While Sobrón had the higher value during the summer (52.5) and La Sotonera in autumn (59.8). The reservoirs during the summer generally presented values between 30 to 50 and during autumn the values increase from 40 to 60. According to Carlson (1996), most reservoirs are classified as mesotrophic, however, Ebro, Oliana and La Sotonera are eutrophic during the autumn and Sobrón during the summer (Fig. 2).

Zooplankton

We registered a total of 40 zooplankton species during both seasons in the six reservoirs (Table 2). The rotifers were the group with more species reported (21), followed by cladocerans (10) and copepods (8). Since the veliger larvae of the zebra mussel invader (*Dreissena polymorpha*) were found at 4 reservoirs, they were considered a separate group inside this study and both abundance and biomass were counted. La Sotonera presented the highest number of species with, 13 in each season, followed by Sobrón with 12 in the summer, Oliana and Mequinenza with 11 during summer. This same species richness was present in Ebro and Ullibarri-Gamboa in the autumn. The lowest number of species was in Sobrón with only 6 during the autumn (Table 2). On average, each reservoir presented 10 zooplankton species per season. The rotifer *Polyarthra dolichoptera* was presented on all reservoirs in at least one season, followed by the zebra mussel, which was detected on four reservoirs during both seasons. The two copepod species *Cyclops vicinus* and *Cyclops* sp., and some rotifers were only presented in one reservoir during one season (Table 2).

The only previous study on these reservoirs was performed during 1987-1988 and reports data of summer and winter seasons. However, to compare species composition per season we only used the summer data from both studies, since the other season is not the same and cannot be compared equally (winter from the previous study and fall in the current). To indicate new registers for each reservoir we verified that species were not present in the data of both seasons from the previous study. The complete list of species present of the previous study can be found in De Manuel & Jaume (1993).

The new registers of zooplankton species for each reservoir are: Sotonera (*Bosmina longirostris*, *Ceriodapnia dubia*, *Ceriodaphnia pulchella*, *Daphnia galeata*, *Diaphanosoma mongolianum*, *Acanthocyclops americanus*, *Anuraeopsis fissa*, *Polyarthra major*, *Ascomorpha ecaudis* and *D. polymorpha*). Ebro (*Eudiaptomus vulgaris*, *B. longirostris*, *D. mongolianum*, *Conochilus unicornis*, *Trichotria tetractis*, *P. major*, *Tricochercca cylindrica*). Mequinenza (*Copidodiaptomus numidicus*, *Thermocyclops dybowskii*, *A. ecaudis*). Sobron (*Daphnia cucullata*, *Cyclops vicinus*, *Asplanchna priodonta*, *P. major*, *A. fissa*) and Oliana (*A. americanus*, *E. vulgaris*, *C. sphaericus*, *D. mongolianum*, *Kellicotia longispina*). Since there is not previous data available for Ullibarri-Gamboa reservoir, all 14 species reported for this study are first register (Table 2).

Table 2. Complete list of zooplankton species found in the six reservoirs. *Listado completo de las especies de zooplancton presentes en los seis embalses.*

	Summer						Autumn					
	ULL	MEQ	EBR	OLI	SOB	SOT	ULL	MEQ	EBR	OLI	SOB	SOT
Cladocera												
Bosmina longirostris	X			X	X	X	X		X	X		X
Ceriodapnia dubia		X		X				X				
Ceriodaphnia pulchella					X	X	X		X	X	X	X
Chydorus sphaericus					X				X	X		
Daphnia cucullata	X				X		X				X	
Daphnia galeata				X		X				X		
Daphnia longispina							X		X			
Daphnia pulicaria			X									
Diaphanosoma mongolianum		X				X		X		X		
Pleuroxus sp.										X		
Copepoda												
Acanthocyclops americanus				X		X	X			X		X
Copidodiaptomus numidicus		X						X				
Cyclops abyssorum			X						X			
Cyclops vicinus					X							
Eudiaptomus vulgaris			X	X					X	X		
Neolovenula alluaudi						X						X
Thermocyclops dybowskii		X						X				
Cyclops sp.											X	
Rotifera												
Anuraeopsis fissa	X										X	X
Ascomorpha ecaudis		X				X		X				
Asplanchna priodonta		X		X		X						
Brachionus angularis	X											
Brachionus calcyflorus					X							
Brachionus havanaensis											X	
Brachionus quadridentatus					X							
Conochilus unicornis	X		X									
Filinia longiseta									X			
Hexarthra fennica								X				
Hexarthra oxyuris							X					
Kellicotia longispina longispina				X								
Keratella cochlearis	X	X	X	X		X	X	X				X
Keratella cochlearis tecta						X	X					X
Keratella quadrata				X								
Polyarthra dolichoptera	X	X	X	X	X	X	X	X	X	X		
Polyarthra major					X		X		X			X
Synchaeta pectinata	X				X	X						X
Synchaeta sp.			X							X		
Trichocerca cylindrica									X			
Trichotria tetractis			X									
Others												
Dreissena polymorpha	X	X			X	X	X	X			X	X

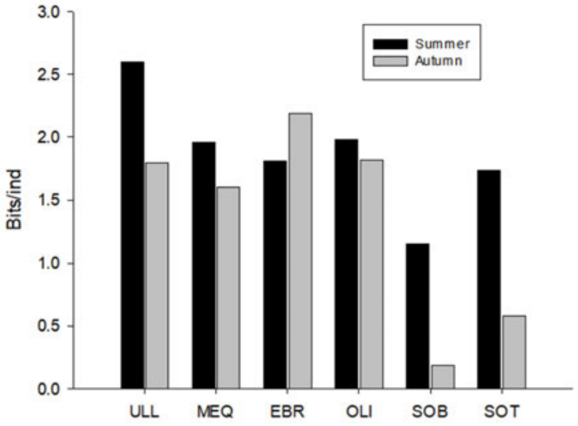

Figure 3. Shannon-Wiener diversity index (black bars represents summer, gray bars autumn). *Diversidad de Shannon-Wiener (barras negras representan verano, grises otoño).*

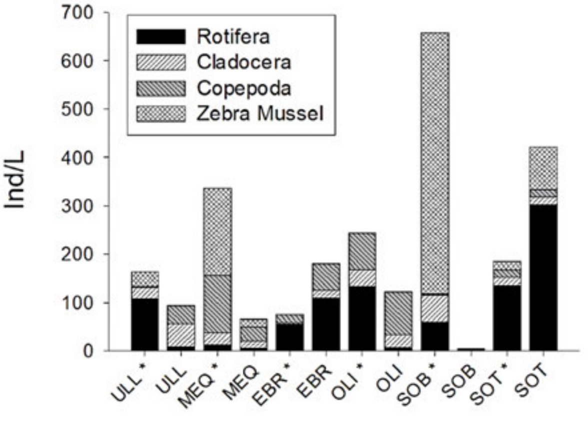

Figure 4. Abundances of zooplankton groups in the six reservoirs, (*) indicate summer values. *Abundancias de los grupos de zooplancton en los seis embalses, (*) indica valores de verano.*

Density and biomass

The zooplankton density varied in each reservoir and season, the average of individuals for all the reservoirs during summer was 277 ind/L, that was higher than in autumn with 148 ind/L. The higher densities in most of reservoir belong to rotifers and zebra mussels, except in Ullibarri-Gamboa, Mequinenza and Oliana during winter (Fig. 4).

In terms of biomass, microcrustaceans have a bigger role instead of rotifers, and each reservoir varied in quantity and group that dominates during both seasons. The reservoirs with major changes between biomass were La Sotonera (dominated during the summer for microcrustaceans to zebra mussels in fall), Sobrón (zebra mussels to cladocerans) and Ullivarri-Gamboa (cladocerans, rotifers and zebra mussels to microcrustaceans mainly) (Table 3). The Shannon-Wiener index indicated that diversity in the Ullibarri-Gamboa reservoir was the highest overall 2.59 bits/ind in summer. The lowest diversity was found in Sobrón during autumn with only 0.18 bits/ind (Fig. 3).

Data analysis

Through the linear Pearson correlations between environmental factors and zooplankton groups we found that pH was significantly correlated with the density of rotifer group (r^2 0.35, $p < 0.05$). Also, both zebra mussel density and biomass were positively correlated with Chl-*a* (r^2 0.60, $p < 0.05$ and r^2 0.62, $p < 0.05$ respectively). Besides, copepods density (r^2 0.39, $p < 0.05$) and biomass (r^2 0.34, $p < 0.05$) were correlated with the reservoir´s depth. Other correlations were not significant ($p > 0.05$). The Analysis of similarity (ANOSIM) doesn't show any difference between both seasons ($p > 0.05$). The contribution of the individual taxa in the dissimilarity of zooplankton was low (SIMPER values < 5 %), being *A. priodonta*, *C. pulchella*, *Synchaeta pectinata* and *D. mongolianum* the responsible for the cumulative of 20 % in the variance of dissimilarity between seasons.

The first CCA, related the physicochemical variables with the principal zooplankton species. The first two axes explains 45.2 % of the variance (p value 0.001 in the Monte Carlo permutation test). Temperature, conductivity and depth are strongly related to copepods (*C. numidicus*, *Cyclops* sp. and *T. dybowskii*) and the cladoceran *C. dubia*. Two of the most abundant rotifers are related with the pH (*S. pectinata* and *C. unicornis*) in addition to the cladoceran *D. cucullata*. A big group composed principally by cladocerans, few copepods and rotifers were related to dissolved oxygen (DO), turbidity, TP, Chl-*a* and suspended solids (SS) (Fig. 5). The rotifer *P. dolichoptera*

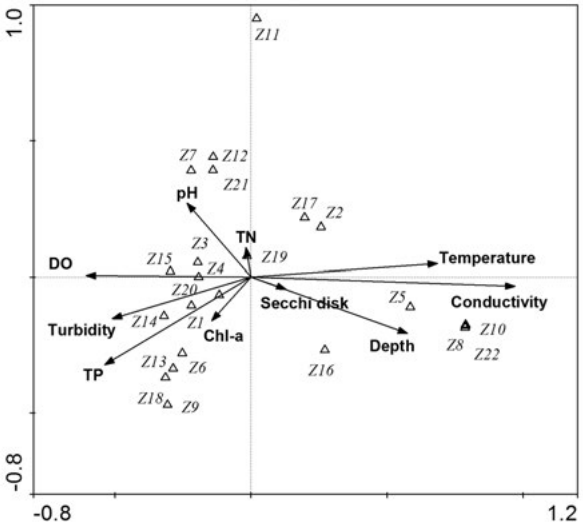

Figure 5. Canonical Correspondences Analysis of the 22 zooplankton main species. DO = Dissolved oxygen, TP = Total phosphorus, TN = Total nitrogen, Chl-*a* = chlorophyll *a*. *Análisis de Correspondencias Canónicas de las 22 especies principales del zooplancton, DO = Oxígeno disuelto, TP = Fosforo total, TN = Nitrogeno total, Chl-*a *= clorofila.* Z1 *Acanthocyclops americanus*, Z2 *Asplanchna priodonta*, Z3 *Bosmina longirostris*, Z4 *Ceriodaphnia pulchella*, Z5 *Ceriodapnia dubia*, Z6 *Chydorus sphaericus*, Z7 *Conochilus unicornis*, Z8 *Copidodiaptomus numidicus*, Z9 *Cyclops abyssorum*, Z10 *Cyclops* sp., Z11 *Cyclops vicinus*, Z12 *Daphnia cucullata*, Z13 *Daphnia galeata*, Z14 *Daphnia longispina*, Z15 *Daphnia pulicaria*, Z16 *Diaphanosoma mongolianum*, Z17 *Dreissena polymorpha*, Z18 *Eudiaptomus vulgaris*, Z19 *Neolovenula alluaudi*, Z20 *Polyarthra dolichoptera*, Z21 *Synchaeta pectinata*, Z22 *Thermocyclops dybowskii*.

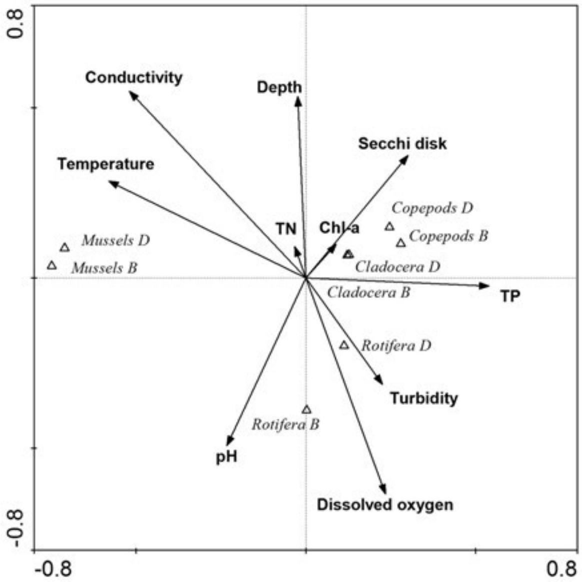

Figure 6. Canonical Correspondences Analysis of the zooplankton groups, D = density, B = biomass, TP = Total phosphorus, TN = Total nitrogen, Chl-*a* = chlorophyll *a*. *Análisis de Correspondencias Canónicas de los grupos del zooplancton, D = densidad, B = biomasa, TP = Fosforo total, TN = Nitrogeno total, Chl-*a *= clorofila.*

was in the middle of the ordination plot, this rotifer was present in all reservoirs during both seasons, their highest abundances were during the summer at La Sotonera (279 ind/L) and Oliana (125 ind/L). The *Daphnia* group was related to DO, TP and turbidity. The *Daphnia* species were present in five reservoirs and their seasonality was split into those which had higher abundances in summer (*D. cucullata*, *D. galeata* and *D. pulicaria*) and in autumn (*Daphnia longispina*). Finally, the copepod *C. vicinus* is not related to any variable and the zebra mussels are slightly connected with pH and alkalinity (Fig. 5).

In the second CCA, we analyzed the environmental variables related with the zooplankton density and biomass, the first two axes represent the most explanatory value (93.2 %) (*p* value > 0.05 in the Monte Carlo permutation test). This CCA indicates that in the first axis the trophic indicators are related (SD, Chl-*a* and TP). These principal indicators are related with both cladocera and copepoda density and biomass, while rotifer density is related to combination of TP, turbidity and DO. The biomass of rotifera group presents a similar relation with pH instead of turbidity. Finally, both density and biomass of zebra mussels are related to temperature and conductivity (Fig. 6).

DISCUSSION

Sommer *et al.* (1986) together with the PEG (Plankton Ecology Group) proposed a model where sequential statements describe the changes in zooplankton and phytoplankton communities in lakes. In these statements, they described that in summer the smaller groups with short generational life cycle dominate and during autumn large species appear. In our study, we found that most of the changes in reservoirs' communities followed these statements, for example, the general tendency at Oliana was that rotifers had high abundances during summer, then, during autumn this group tended to decrease and microcrustaceans increased in number becoming the dominant group. Ullibarri-Gamboa presented a similar tendency but with higher abundances of rotifers and mussels during the summer, followed by the increase of copepods and cladocerans during autumn. At the Ebro and La Sotonera reservoirs, the number of all groups increase in autumn but with the rotifers being the dominant group. However, at Mequinenza the zebra mussels and copepods were dominants during summer reaching up 350 ind/L, but with a decrease during autumn. Sobrón shows a similar tendency, the summer was dominated by the zebra mussel (540 ind/L) and in the next period densities of all groups decreased dramatically. For these two last reservoirs several factors could explain these changes, such as an extreme fish predation (Amundsen *et al.*, 2009; Ginter *et al.*, 2019), the establishment of the sessile stage of mussels in any surface decreasing the number of the planktonic larvae (Claudi & Mackie, 1994) or even some criteria that were not taken in count in the previous model, such as the food quality and the trophic level of each reservoir (Sommer *et al.*, 2012).

Biodiversity is strongly related with environmental factors (Jeppesen *et al.*, 2000), while some physiochemical parameters such as temperature, dissolved oxygen, pH, etc., can have positive or negative effects on zooplankton (Wetzel, 2001). One of the more efficient analyses to correlate the zooplankton communities with the physical and chemical variables is the CCA (Attayde & Bozelli, 1998). Data from our CCA analysis shows the rotifer *P. dolichoptera*, which was positioned in the middle of the ordination plot, due to their high tolerance to different environments conditions (Bērziņš & Pejler, 1989), nowadays it has a wide distribution in many water bodies around the world (Segers, 2007). The copepod *Neolovenula alluaudi*, that is typically from the Mediterranean area (Miracle, 1982), also was positioned near the center of the CCA. We can infer that they possess high tolerance, however, compared to the previously mentioned rotifer, it was only present at La Sotonera reservoir. The populations of this copepod are moving from the south and are now found in several water bodies along the Iberian Peninsula (Alfonso & Belmonte, 2013; Miracle, 1982). Thus, this copepod was reported at Mequinenza 30 years ago (De Manuel & Jaume, 1993), but not found during the present study. Furthermore, at the Mequinenza reservoir the presence of silurids is well documented and the early stages of this fish can consume copepods and large cladocerans individuals as the *Daphnia* species, they can promote the small-size species such as *C. dubia* and *D. mongolium*, (Miranda *et al.*, 2010). Also, in this reservoir no *Daphnia* species were recorded, probably due the combination of predation and lower levels of oxygen compared to other reservoirs (Hanazato, 1996).

The copepods *C. numidicus* and *T. dybowskii* were correlated with conductivity, temperature and depth. It is well known that big-sized zooplankton species perform a daily vertical migration to avoid depredation (Hays, 2003; Lampert, 1989). The study of Caramujo & Boavida (2000) found that these two copepod species can be consumed in large numbers by fishes, for this reason, their populations are settled in deepest water bodies. In this study, we found both species only at Mequinenza, which has an average 50 m of depth in both seasons. The biggest copepod found in this study was *C. numidicus* and it provides a high percentage of total biomass and density of all copepods, thus, Pearson correlation was significative in terms of depth for this group.

In the CCA for groups (Fig. 6) the rotifer biomass was also correlated to pH, other studies have shown that this parameter can affect the rotifer occurrence (Bērziņš, 1987) and their assemblage in reservoirs (Devetter, 1998).

The complex of abundances and biomasses of microcrustaceans (copepods and cladocerans), were related with the components that conform the trophic state since they are influenced by the Secchi disk, TP and Chl-*a*. Some authors have indicated that large species of these groups can be used as an indicator of oligotrophic state (Pejler, 1983; Moss *et al.*, 2003; Kane *et al.*, 2009; Haberman & Maldna, 2014). Usually, at higher trophic level, large species are replaced by small species (Lampert & Sommer, 1997). The Ebro, Oliana and La Sotonera reservoirs, during the autumn, were mesotrophic to eutrophic, and densities of larger species as *Daphnia* spp. decay while smaller cladocerans such as *B. longirostris* and *Ceriodaphnia* spp. increase.

Despite the limitations of this work (and taking in count the low number of reservoirs sampled compared with the watershed size), with the data obtained from the CCA we could hypoth-

Table 3. Density (ind/L), Biomass (mg/L) and their percentage (%) of zooplankton groups present on the six reservoirs. *Densidad (ind/L), Biomasa (mg/L) y el porcentaje (%) de los grupos del zooplancton presentes en los seis embalses.*

	Summer				Autumn			
	ind/L	mg/L	ind/L %	mg/L %	ind/L	mg/L	ind/L %	mg/L %
Ullibarri-Gamboa								
Cladocerans	23.46	31.15	14.4	47.14	47.12	95.06	49.8	73.95
Copepods	2.31	0.34	1.42	0.52	38.85	32.87	41.06	25.57
Rotifers	107.69	22.23	66.12	33.64	8.27	0.47	8.74	0.36
Mussels larvae	29.42	12.35	18.06	18.7	0.38	0.16	0.41	0.13
Total	162.88	66.07	100	100	94.62	128.55	100	100
Mequinenza								
Cladocerans	24.62	47	7.31	11.3	15	29.54	22.61	36.75
Copepods	118.08	287.74	35.05	69.19	29.81	43.88	44.93	54.59
Rotifers	13.08	5.03	3.88	1.21	5.77	0.33	8.7	0.41
Mussels larvae	181.15	76.08	53.77	18.3	15.77	6.62	23.77	8.24
Total	336.92	415.86	100	100	66.35	80.37	100	100
Ebro								
Cladocerans	3.85	14.62	5.12	23.46	16.73	29.96	9.25	23.29
Copepods	15.96	42.38	21.23	68.04	55.19	91.83	30.5	71.39
Rotifers	55.38	5.29	73.66	8.5	109.04	6.83	60.26	5.31
Total	75.19	62.29	100	100	180.96	128.62	100	100
Oliana								
Cladocerans	34.8	75.9	14.22	44.69	24.81	36.71	20.31	48.05
Copepods	76.92	86.86	31.42	51.14	89.42	39.29	73.23	51.43
Rotifers	133.08	7.09	54.36	4.17	7.88	0.4	6.46	0.52
Total	244.8	169.85	100	100	122.11	76.4	100	100
Sobrón								
Cladocerans	54.23	104.17	8.24	29.75	0.77	1.62	19.05	64.52
Copepods	3.08	3	0.47	0.86	1.73	0.35	42.87	13.83
Rotifers	60.58	16.21	9.21	4.63	0.38	0.06	9.53	2.32
Mussels larvae	540	226.8	82.08	64.77	1.15	0.48	28.55	19.34
Total	657.88	350.19	100	100	4.04	2.5	100	100
Sotonera								
Cladocerans	16.92	24.19	9.19	39.5	15.77	24.15	3.74	23.44
Copepods	16.73	20.68	9.08	33.77	15.58	25.92	3.7	25.16
Rotifers	134.62	9.67	73.07	15.78	302.5	16.29	71.83	15.81
Mussels larvae	15.96	6.7	8.66	10.95	87.3	36.67	20.73	35.59
Total	184.23	61.24	100	100	421.15	103.03	100	100

esize the zooplankton groups, such as, copepods and cladocerans could be affected firstly and their structure modified if the variables that are more related or affect these groups change for several factors, such as, climate change, new invasive species and or anthropogenic impacts.

The zooplankton community normally varied through months, seasons or years, and the species replacement can happen quickly or change gradually with time (Lampert & Sommer, 1997). Some of these species' substitutions can be observed in the current research compared with data of previous works, at Mequinenza, from species reported previously for summer season we found only two shared species. Larger filter species such as *N. alluaudi* and *D. galeata* together with the main predator *A. robustus*, were substituted for *C. numidicus* and *T. dybowskii*. A similar case occurred at Sobrón, where only three species were shared. From three cyclopids species to only *C. vicinus* and the presence of *D. galeata* and *C. pulchella*.

The reservoir with the most shared species was Oliana, with seven of the nine species reported for this study. The main change observed was *C. abyssorum* to *A. americanus*. The Sotonera reservoir was the only reservoir where the two previous copepod species did not suffer any variation, however, cladocerans from two *Daphnia* species changed to one species (*D. galeata*) and medium-size filters as *C. dubia* and *D. mongolianum*. The study of Higgins & Vander Zanden (2010) suggests that *D. polymorpha* can reduce the zooplankton biomass to 40-77 % in pelagic areas and replace them, this affect the species richness and diversity. The low replacement at Oliana could be related to the non-presence of them. In contrast, some changes can be appreciated at La Sotonera and Sobrón with low diversity (Fig. 3) for their increase in density and biomass (Table 3).

For the Ebro reservoir, there is a great difference in the number of zooplankton species between studies since previously 20 species were reported, where almost half of the species were microcrustaceans including several species of *Daphnia* and cyclopids, however, we registered only four shared species. Nowadays, only eight species are present, where five of them belong to rotifers and only *D. pulicaria* and *C. abyssorum* were reported before. Thus, all these data indicate that the communities have changed, increasing, or decreasing the number of species and being replaced for others. Several explanations such as competition, natural succession or even variations of environmental variables (Devetter, 1998; Dodson *et al.*, 2009) could explain these changes, however, since there is a lack of information for all non-reported years, the question of which exact events caused these changes remains unanswered.

Due to diverse factors, including management, most of water bodies cannot be sampled on a regular basis to confirm the species presents and like in this study, can take a long time until having new data. Nevertheless, having a monitoring program could help us to understand the community changes. But this is not the only benefit, thus, it can be a tool to have complete knowledge of species richness and to identify the already reported and the newly invasive species. For the invasive fauna, correct actions could prevent their introduction and dispersal along the watershed area, which could not only affect local diversity and become one of the major aquatic stressors, as is the case with zebra mussels (Strayer, 2010), but also create economic losses due to their impact on important infrastructures (Duran *et al.*, 2012).

The Zebra mussels were detected for first time at the Ebro watershed in 2001 (Duran & Anadón, 2008). Previously at La Sotonera reservoir the presence of *D. polymorpha* was not detected, however, now the veliger larvae can be found at both seasons and it's a dominant component of zooplankton. Thus, the mussel invasion has progressed throughout the years and among different reservoirs. The two reservoirs were mussels were not present are Oliana and Ebro, this last is under special protection (Duran & Anadón, 2008). Due to the lack of natural predators, efficient competition and non-intentional dispersion of invaders caused by the interaction between people among the reservoirs in the area, this invader could be detected in the Ebro reservoir in the upcoming years. Consequently, they would be present from the beginning until the end of watershed.

CONCLUSION

Our results show that abundances and biomass values were in general two times higher in summer than values in autumn. However, there is not an equal tendency for all reservoirs and each one works in a different way. The data suggest that the changes in the zooplankton community during both seasons are related mainly with physico-chemical variables as Chl-*a*, SD, TP, pH and reservoir depth, as well as with biotic interactions, like competition with alien species such as *D. polymorpha*. The relation between the zooplankton groups and the environmental variables could help us understand the main changes that could occur in a shifting world. All reservoirs presented new records in zooplankton species. La Sotonera had the highest number of new registers with ten species, followed by the Ebro with seven, while Oliana, Sobrón and Mequinenza have five. For Ullibarri-Gamboa reservoir we showed for the first time a record of zooplankton species. Also, we detected for first time the presence of zebra mussels at La Sotonera reservoir, indicating that this invader is dispersing throughout the watershed. Therefore, zooplankton composition knowledge, regular monitoring of species inhabiting in the reservoirs and the understanding of environmental variables that affect species and zooplankton structure (specific richness, density and biomass) can be a helpful tool for watershed management and early detection of invasive species.

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Zooplankton of Cúber and Gorg Blau reservoirs, Majorca (Mallorca). An unpublished contribution by Dr. Maria Rosa Miracle to the regional limnology of the Balearic Islands

Maria Rosa Miracle[1,†], Guillermo Ramón[2] and Gabriel Moyà[2,*]

[1] Institut Cavanilles de Biodiversitat i Biologia Evolutiva. Universitat de València.
[2] Departament de Biologia, Universitat de les Illes Balears, Carretera de Valldemossa, km 7.5, 07122 Palma de Mallorca (Balears).

[*] Corresponding author: biel.moya@uib.cat

ABSTRACT

Zooplankton of Cúber and Gorg Blau reservoirs, Majorca (Mallorca). An unpublished contribution by Dr. Maria Rosa Miracle to the regional limnology of the Balearic Islands

Unpublished zooplankton results from some samples of Cúber and Gorg Blau reservoirs are presented. The samples are collected during the years 1976 and 1977, and studied by Dr. Maria Rosa Miracle. These are the only zooplankton community samples analyzed from these reservoirs, during their almost forty years of operation and that, they were stored for more than 30 years before their study by Dr. Miracle. This article, beyond its possible scientific interest, wants to be a tribute to Dr. Miracle, and demonstrates once again her rigor in systematic studies, and her interest and curiosity for any sample from nature habitats, no matter how small it was. The results show the greatest diversity of rotifers at Cúber reservoir, and slightly higher maximum densities of organisms at Gorg Blau reservoir.

Key words: zooplankton, reservoirs, Majorca (Mallorca) islands

RESUMEN

Zooplancton de los embalses de Cúber y Gorg Blau (Mallorca). Una contribución inédita de la Dra. Maria Rosa Miracle a la limnología regional de les Illes Balears

Se presentan los resultados inéditos de unas muestras del zooplancton de los embalses de Cúber y Gorg Blau, recogidas durante los años 1976 y 1977, y estudiadas por la Dra. Maria Rosa Miracle. Son las únicas muestras de las comunidades zooplanctónicas de dichos embalses analizadas hasta ahora, después de casi cuarenta años de la puesta en funcionamiento de dichos embalses y de que las mismas estuvieron más de 30 años almacenadas antes de su estudio por la Dra. Miracle. Este artículo, más allá de su posible interés científico, quiere ser un homenaje a la Dra. Miracle, y demuestra una vez más su rigor en los estudios sistemáticos y su interés y curiosidad por cualquier muestra de hábitats naturales, sin importar lo pequeña que esta fuera. Los resultados ponen de manifiesto la mayor diversidad de rotíferos en el embalse de Cúber, y unas densidades máximas de organismos ligeramente superiores en el embalse del Gorg Blau.

Palabras clave: *zooplancton, embalses, Mallorca*

INTRODUCTION

Cúber and Gorg Blau are two reservoirs located at "Serra de Tramuntana" on Majorca island, connected by a channel that collects the deep waters of Gorg Blau and discharges them into Cúber surface waters. Both have a warm monomictic dynamic (Ramón & Moyà, 1983), and present oxygen deficit in the hypolimnion during summer months (Moyà & Ramón, 1984). With volumes of 4.6 and 6.9 hm^3, both came into service in 1972, with the aim of completing the water supply to the city of Palma. They have maximum depths of 17 and 35 m, average depths of 7.76 and 11.55 m, water renewal rates of 8 and 4 hm^3/year and the surfaces of the respective reception basins are 8.5 and 7.4 km^2 (Ramón & Moyà, 1982). As indicated in Table 1, the waters of both reservoirs can be classified as mineralized. While the average concentration of nitrogen in the form of nitrates is higher at Cúber reservoir, the value of chlorophyll *a* is slightly higher at Gorg Blau.

MATERIAL AND METHODS

Zooplankton samples were obtained by dragging a tow plankton net, 40 micrometers mesh size, from a paddle dinghy, during a towing time of 15 minutes, in the central and deepest part of both reservoirs. Once collected, the samples were fixed with a 4 % solution of formaldehyde.

Although the first study of these reservoirs covered the period from 1976 to 1978, the zooplankton samples analyzed by Dr. Miracle only correspond to the period of August 1976 to September 1977 for Cúber reservoir, and June 1977 for Gorg Blau.

All zooplankton samples results have been tabulated exactly as prepared by Dr. Miracle. After the analyses have been performed, the listings do not include any additional comments.

RESULTS AND DISCUSSION

Tables 2 and 3 present the results of qualitative and quantitative analyses of zooplankton in Cúber and Gorg Blau, carried out on samples collected during routine sampling of physicochemical and biological variables in these reservoirs.

For the Cúber reservoir, the classification of zooplankton taxa includes the following groups: cladocerans, copepods, rotifers, and a fourth group without taxonomic category that brings together different types of organisms that were collected by the tow nets and that in general, with the exception of ostracods and tecamebas, are little studied in the routine analyses of the zooplankton. For the Gorg Blau reservoir the corresponding table only includes cladocerans, copepods and rotifers.

Other components of zooplankton in the reservoirs of Cúber and Gorg Blau, which are not listed in Tables 2 and 3, only observed in point samples, also analyzed by Dr. Miracle, are Cladocera: *Daphnia longispina*, *Ceriodaphnia quadrangula*, present in both reservoirs, *Leydigia quadrangularis*, only in Cúber. Copepods: *Macrocyclops distinctus*, *Eucyclops serrulatus*, only in Cúber. Ostracods: *Cypridopsis newtoni*, only in Cúber. Rotifers: *Asplanchna priodonta*, only in Cúber, *Polyarthra vulgaris*, in both reservoirs.

The lists of rotifers show notable differences between the two reservoirs. Their number is three

Table 1. Extreme and average values of some of the chemical variables and the concentration of chlorophyll a, over the period 1976 to 1978, at Cúber and Gorg Blau reservoirs (Majorca). *Valores extremos y medios de algunas de las variables químicas y de la concentración de clorofila a, a lo largo del período 1976 a 1978, en los embalses de Cúber y Gorg Blau (Mallorca).*

Variable	Alkalinity Meq/L	Calcium Mg/L	PO_4^{3-} µM	NO_3^- µM	Chlorophyll *a* µg/L
Cúber	1.10-3.10	28.00-112.00	0.03-2.2	0.00-45.00	2.70-8.40
	2.40	74.70	0.38	4.53	3.70
Gorg Blau	1.90-4.30	48.00-120.00	00.3-1.60	0.00-45.00	2.30-9.90
	2.72	77.00	0.40	2.25	4.08

Table 2. List and abundances of the different taxa determined at zooplankton samples from Cúber reservoir (Majorca). *Listado y abundancias de los diferentes taxones determinados en las muestras de zooplancton del embalse de Cúber (Mallorca).*

Reservoir and Date	**Number of individuals/L (Relative abundance)**					
	Cúber 1-C 19/08/76	Cúber 19/08/76	Cúber 15/03/77	Cúber 24/05/77	Cúber 21/06/77	Cúber 27/09/77
Cladocerans						
Ceriodaphnia pulchela	65 (11.99)	73 (13.57)				19 (4.00)
Chydorus sphaericus	3 (0.55)	3 (0.55)	19 (3.54)	5 (0.73)	11 (2.08)	
Daphnia galeata	40 (7.38)	49 (9.11)	442 (82.46)	582 (85.46)	118 (22.29)	160 (33.68)
Simocephalus vetulus	60 (11.07)	100 (18.59)				
Copepoda						
Macrocyclops albidus						
♀	1 (0.18)	4 (0.74)		5 (0.73)	5 (0.09)	
♂	2 (0.37)	2 (0.37)	1 (0.19)	1 (0.15)		
Copepoditos cyclópido	87 (16.05)	76 (14.13)	4 (0.75)	39 (5.73)	1 (0.19)	
Nauplios cyclópido	93 (17.16)	100 (18.59)	2 (0.37)	35 (5.14)	10 (1.89)	20 (4.21)
Rotifera						
Anuraeopsis fissa	1 (0.18)	3 (0.56)			8 (1.51)	59 (12.42)
Ascomorpha saltans	1 (0.18)		5 (0.93)			
Asplanchna girodi	3 (0.55)	6 (1.12)				
Bdelloidea	38 (7.01)	19 (3.53)				
Cephadella megalocephala	1 (0.18)					
Collotheca cf pelagica	4 (0.74)	7 (1.30)			6 (1.13)	59 (12.42)
Colurella uncinata	14 (2.58)	9 (1.67)		4 (0.59)	28 (5.29)	
Colurella uncinata bicuspidata				1 (0.15)	2 (0.38)	1 (0.21)
Lecane closterocerca	14 (2.58)	4 (0.74)			3 (0.57)	
Lecane furcata	1 (0.18)	2 (0.37)				
Lecane lunaris	4 (0.74)	1 (0.19)		1 (0.15)		1 (0.21)
Lepadella patella	3 (0.55)					
Polyarthra longiremis			40 (7.46)			
Polyarthra cf luminosa	23 (4.24)	22 (4.09)			10 (1.89)	29 (6.11)
Pleurotrocha cf petromyzon		6 (1.12)				
Synchaeta oblonga			14 (2.61)	1 (0.15)		
Synchaeta cf kitina						44 (9.26)
Testudinella patina					2 (0.38)	
Trichocerca insignis						3 (0.63)
Trichocerca cf pusilla	1 (0.18)					
Trichocerca rattus carinata					1 (0.19)	
Trichocerca cf tenuior	7 (1.29)	2 (0.37)				
Trichocerca similis	23 (4.24)	3 (0.56)		1 (0.15)	324 (61.19)	79 (16.63)
Filina longiseta (huevo)			1 (0.19)		1 (0.19)	
Others						
Mites	4 (0.74)	2 (0.37)			1 (0.19)	1 (0.21)
Chironomids					1 (0.19)	
Diptera	1 (0.18)					
Ephemeral	33 (6.09)	36 (6.69)	2 (0.37)	6 (0.88)	1 (0.19)	
Nematodes		1 (0.19)	2 (0.19)			
Oligochetes			1 (0.19)			
Ostracods	3 (0.55)	4 (0.74)	2 (0.37)		1 (0.19)	
Tecate amoebas	12 (2.21)	4 (0.74)	1 (0.19)			
Total	542	538	536	681	530	475

times higher in Cúber, where species diversity is high. Besides the eight taxa present in the Gorg Blau, half of them are not found in Cúber.

Given the proximity between these two reservoirs and their almost simultaneous commissioning, the unequal richness of rotifers between the two is astonishing a priori. We put forward two hypotheses, which together could explain the difference observed. The presence in Cúber of rotifers whose preferential habitat is coastal vegetation or even the benthos- as for the different species of *Lecane* and *Trichocerca* (Ruttner-Kolisko, 1974)- would be due to the shallower depth maximum and average depths of this reservoir, and to a much shallower relative depth factors which would have a greater effect because the proximity to the bottom of the tow nets.

Another hypothesis that complements the previous one is the different trophic status of these reservoirs. During the period in which the zooplankton was sampled, the phytoplankton community at Gorg Blau reservoir was dominated by the cyanobacterium *Planktothrix (Oscillatoria) rubescens*, whose filaments are poorly eaten by the different zooplankton taxa (Margalef, 1983). A low N/P ratio in the waters of Gorg Blau, favored the monoculture of cyanobacteria during the first years of the reservoir operation (Ramón & Moyà, 1984), a situation which changed over time as that ratio increased (Moyà *et al.*, 1993). The phytoplankton community of the Cúber reservoir was more complex and diverse, seasonal changes followed the theoretical model of annual succession, and the incidence of the incoming *Planktothrix* filaments from Gorg Blau was insignificant (Moyà & Ramón, 1984).

A study on the presence of rotifers in different epicontinental aquatic ecosystems, both temporary and permanent, on Majorca Island (De Manuel, 1989, 1990) gives a figure of 33 species, a number which practically coincides with the 34 species determined in Dr. Miracle's study at Cúber and Gorg Blau reservoirs. However, significant differences are observed when comparing the lists of rotifers in these two reservoirs with that of the study carried out for the whole island. For both reservoirs, only half of the taxa coincides with those determined for the whole of Majorca. The absence of typical species of coastal ecosystems, of brackish water, in the reservoirs, together with the lack of some common rotifers in

Table 3. List and abundances of the different taxa determined at zooplankton samples from Gorg Blau reservoir (Majorca). *Listado y abundancias de los diferentes taxones determinados en las muestras de zooplancton del embalse de Gorg Blau (Mallorca).*

Reservoir Date	Number of individuals/L (Relative abundance)					
	Gorg Blau 05/08/76	Gorg Blau 16/11/76	Gorg Blau 12/04/77	Gorg Blau 1 07/06/77	Gorg Blau 2 07/06/77	Gorg Blau 06/12/77
Cladocera						
Alona rectangulare			1 (0.23)			+
Ceriodaphnia pulchela	1 (0.41)	295 (48.52)	1 (0.23)	4 (0.39)	5 (0.64)	
Chydorus sphaericus						+
Daphnia galeata		292 (48.03)	424 (95.71)	541 (52.74)	422 (54.38)	
Copepoda						
Copepoditos cyclópido		1 (0.08)				
Nauplios cyclópido		1 (0.08)		1 (0.10)	1 (0.13)	
Rotifera						
Bdelloidea			1 (0.23)	1 (0.10)	1 (0.13)	
Euchlanis dilatata				2 (0.19)		
Lecane closterocerca		1 (0.16)				
Lecane luna			1 (0.10)			
Polyarthra cf dolichoptera		6 (0.99)	11 (2.48)	6 (0.58)	1 (0.13)	
Synchaeta cf kitina				19 (1.84)	12 (1.55)	
Trichocerca similis	240 (99.59)	12 (1.97)	5 (1.13)	456 (44.23)	333 (42.91)	
Trichocerca tetractis		1 (0.16)			1 (0.13)	
Total	241	608	443	1031	776	0

permanent water ecosystems such as *Keratella* or *Hexarthra*, justify the observed differences.

The number of taxa observed in both reservoirs is minimal for copepods, only three species in Cúber and only unspecified nauplii and copepodites in Gorg Blau. The number of Cladocera is slightly higher at Cúber reservoir, 7 taxa vs. 6 in Gorg Blau, but some of the species present are different: *Simocephalus vetulus* and *Leydigia quadrangularis* are found only in Cúber and *Alona rectangulare* only in Gorg Blau.

With regard to Cladocera, it is interesting to note that the most abundant species in both reservoirs is *Daphnia galeata*, which, according to the work of Armengol (1978), was only found at two reservoirs in the final Ebro river stretch, but that, based on the review carried out by Jaume (1993), a much wider distribution is attributed to it in the set of Iberian Peninsula reservoirs and, in addition, it is also an abundant species in lakes of central and northern Europe. It is probably that, like most zooplankton species, the arrival and colonization of these two reservoirs in Majorca occurred through migratory birds. One aspect to point out in relation to the possible inhibition of *Daphnia* populations by the cyanobacterium *Planktothrix* (Infante & Abella, 1985), is that the maximum densities of *D. galeata* were recorded throughout the spring and early summer, when the stratification of the water column began and the bulk of the *Planktothrix* population was concentrated at the reservoir metalimnion, staying the epilimnion free of filaments (Ramon & Moyà, 1984). But it should also be noted that, according to the work of Jaume (1993), *D. galeata* is a macro-filter, which gives him an advantage over other components of zooplankton with the same trophic strategy.

Given the limited information available, we cannot draw conclusions about the dynamics of zooplankton or, in general, about other aspects related to its ecology in the two presented reservoirs. Nevertheless, it should be noted that the two taxa with the highest densities, which are dominant in the zooplankton communities of Cúber and Gorg Blau, are *Daphnia galeata* and *Trichocerca similis*. Therefore, as is the case for all the studied reservoirs of the Iberian Peninsula (Jaume, 1993), cladocerans are the dominants in the communities of both reservoirs.

By way of conclusion on the zooplankton study in the reservoirs of Cúber and Gorg carried out by Dr. Miracle, we would like to insist on the merits of her work because it is the first and so far the only contribution to the knowledge of the zooplanktonic communities of these two permanent water bodies of the Majorca Island. Furthermore, it was carried out during the first stages of its colonization by plankton organisms, and, despite of this, with a high diversity of species, mainly at Cúber reservoir. We hope that this work will be a stimulus for future research on the limnology of Majorca reservoirs and in particular for a better understanding of their zooplankton. We believe that it would be the best tribute to the memory of Dr. Maria Rosa Miracle.

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We are ready for faunistic surveys of bdelloid rotifers through DNA barcoding: the example of *Sphagnum* bogs of the Swiss Jura Mountains

Diego Fontaneto[1,*], Ester M. Eckert[1], Nikoleta Anicic[1], Enrique Lara[2,3] and Edward A. D. Mitchell[2,4]

[1] National Research Council of Italy, Water Research Institute, Microbial Ecology Group, largo Tonolli 50, I-28922 Verbania Pallanza, Italy.
[2] Laboratory of Soil Biodiversity, Institute of Biology, University of Neuchâtel, Rue Emile-Argand 11, CH-2000 Neuchâtel, Switzerland.
[3] Real Jardín Botánico, CSIC, Plaza Murillo 2, ES-28014 Madrid, Spain.
[4] Jardin Botanique de Neuchâtel, Chemin du Perthuis-du-Sault 58, CH-2000 Neuchâtel, Switzerland.

[*] Corresponding author: diego.fontaneto@cnr.it

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ABSTRACT

We are ready for faunistic surveys of bdelloid rotifers through DNA barcoding: the example of *Sphagnum* bogs of the Swiss Jura Mountains

The identification of biological diversity through DNA barcoding and metabarcoding of the organisms living in the field has the potential to revolutionise the way biological surveys and monitoring are performed. Yet, we still do not know if the current representativeness of the reference database of DNA sequence data is sufficient to allow such approaches. Here, we show that, at least for bdelloid rotifers (Metazoa; Rotifera; Bdelloidea) in Europe, current knowledge is ripe to perform such surveys. We show the results of an exercise performed on bdelloid rotifers in *Sphagnum* bogs of the Swiss Jura Mountain. The results of DNA-based identifications were rather consistent with the morphology-based identifications, and the few cases of mismatch could be used as a cautionary tale to avoid potential misinterpretations of results. The mismatches were due to cases of the closest match not being genetically very close, and to the occurrence of cryptic species.

Key words: Bdelloidea, biodiversity, cytochrome c oxidase subunit I, Rotifera, taxonomy

RESUMEN

Listos para revisiones faunísticas de rotíferos bdelloideos por DNA barcoding*: el caso de las turberas de* Sphagnum *de las montañas del Jura suizo*

La identificación de la diversidad biológica a través de DNA barcoding *y* metabarcoding *de los organismos en el medio ambiente tiene el potencial de revolucionar la forma en que se realizan los inventarios biológicos y el monitoreo. Sin embargo, todavía no se sabe si las bases de datos genéticos de referencia a disposición hoy en día son lo suficientemente representativas como para permitir tales enfoques. Aquí, mostramos que, al menos para los rotíferos bdelloideos (Metazoa; Rotifera; Bdelloidea) de Europa, el nivel de conocimiento es suficiente para realizar tales estudios. Mostramos los resultados de un ejercicio realizado sobre rotíferos bdelloideos en turberas de* Sphagnum *del Jura suizo. Los resultados de las identificaciones basadas en el ADN fueron bastante consistentes con las identificaciones basadas en la morfología, y los pocos casos de desajuste podrían utilizarse como una advertencia para evitar posibles interpretaciones erróneas de los resultados. Estos desajustes se debieron a que las secuencias más cercanas seguían alejadas de los organismos realmente encontrados y a la presencia de especies crípticas.*

Palabras clave: *Bdelloidea; biodiversidad, subunidad I de la citocromo c oxidasa, Rotifera, taxonomía*

INTRODUCTION

Rotifers are one of the most common and abundant groups of animals living in continental waters (Fontaneto & De Smet, 2015). The known global richness of this phylum is not very high, with only slightly more than 2000 species described (Segers, 2007); on the other hand, local richness can be quite high, with more than 100 species occurring in a single temperate lake (Dumont & Segers, 1996; Segers & De Smet 2008). The geographic distributions of species are very wide, allowing comparisons of communities in similar ecosystems across continents (Fontaneto *et al.*, 2012). Given their ubiquity and abundance, rotifers have been suggested as useful biomonitors of environmental quality (Sládeček, 1983; Obertegger *et al.*, 2011; Kuczyńska-Kippen, 2018). Yet, their routine identification is hampered by a high degree of phenotypic plasticity in several morphological features (Gilbert, 2017) coupled with a high degree of morphological stasis for other features (Campillo *et al.*, 2005). Such taxonomic uncertainty is mirrored in the high degree of cryptic species found to date in all groups for which DNA sequences are available (García-Morales & Elías-Gutiérrez, 2013; Mills *et al.*, 2017; Kordbacheh *et al.*, 2017), with the further complication of between-species hybridisation (Suatoni *et al.*, 2006; Papakostas *et al.*, 2016; Obertegger *et al.*, 2018). Finally, as morphological identification often requires observing living specimens to see the necessary identification criteria, especially for bdelloid rotifers (Donner, 1965), samples cannot be fixed. Using DNA extracted from environmental samples (eDNA) could be a useful alternative solution for practical applications of rotifers as bioindicators, but this approach has not yet been validated.

Thanks to several studies on DNA taxonomy and population genetics in rotifers, many DNA sequences are nowadays available for several species: a GenBank search performed on February 16th 2018 gave an astounding figure of almost 10 000 hits in Rotifera for cytochrome c oxidase I (COI), the most commonly used barcoding marker to date for animals (Hebert *et al.*, 2003). Thus, such a marker could be used to obtain information on the occurrence of species in the field, bypassing the need for a morphological approach for species identification by directly obtaining DNA sequence data to be compared with a reference database. Such an approach is what several researchers are advocating for the future of biological monitoring (Leese *et al.*, 2018), called biomonitoring 2.0 (Baird *et al.*, 2012). The identification of single organisms through DNA sequences from a reference marker is an established approach, called DNA barcoding (Hebert *et al.*, 2003). Its extension at the community level (the identification of the whole group of organisms living in a sample) is called DNA metabarcoding (Taberlet *et al.*, 2012), and is considered at the forefront of biomonitoring 2.0 (Leese *et al.*, 2018).

One of the problems of applying such an approach in the field for routine biological monitoring and faunistic studies is that we still do not know if a DNA-based identification would be applicable to rotifers, because of the presence of cryptic species and of how far the existing reference database would be exhaustive enough to provide accurate taxonomic assignments. For well-studied animals such as fish (Díaz *et al.*, 2016), Lepidoptera (Huemer *et al.*, 2014), Ephemeroptera, Plecoptera, and Trichoptera (Morinière *et al.*, 2017), the reference databases seem reliable, but for understudied microscopic animals the reliability needs to be demonstrated. The aim of this study is to provide an example of such an application of DNA barcoding, testing its efficiency and assessing its potential biases. In order to produce a reliable test of a faunistic study performed on DNA sequence data, we focused on one group of rotifers, the bdelloids, for which taxonomic uncertainties are high (Fontaneto *et al.*, 2009) and faunistic studies are scarce. Moreover, we performed field sampling in Switzerland, where rotifer diversity is highly understudied: no records of bdelloids are available for the country in the Fauna Europaea database (de Yong *et al.*, 2014). Therein, we focused on a highly specific and understudied habitat, *Sphagnum* bogs, because the diversity of bdelloids is known to be relatively high in small water bodies with acidic waters (Donner, 1965).

The rationale of the tests included a comparison between morphological identification and

Table 1. List of samples analysed during the project. Acronyms for localities are: NE, Neuchâtel; JU, Jura; BE, Bern). Coordinates are in the WGS84 reference system. *Lista de muestras analizadas durante este proyecto. Los acrónimos para las localidades son: NE, Neuchâtel, JU, Jura; BE, Bern). Las coordenadas están en el sistema de referencia WGS84.*

Sample code	Locality	Description	Date	Habitat	North	East
D01	Le Cachot (NE)	Fossé Pochon (former peat extraction ditch)	07/10/14	wet *Sphagnum cf. fallax*	47.004665°	6.664461°
D02	Bois des Lattes (JU)	Pool in a secondary (cutover) part of bog	07/10/14	wet/submerged *Sphagnum* spp.	46.973649°	6.708459°
D03	Le Cachot (NE)	Large *Sphagnum cuspidatum* pool in the centre of the bog	07/10/14	wet/submerged *Sphagnum cuspidatum*	47.005440°	6.665511°
D04	Bois des Lattes (NE)	Blocked drainage ditch with *Carex rostrata*, *Sphagnum cf. fallax*	07/10/14	wet/submerged *Sphagnum cf. fallax*	46.972924°	6.707294°
D05	Le Cachot (NE)	Side of a large *Sphagnum cuspidatum* pool in the centre of the bog	07/10/14	*Sphagnum magellanicum*	47.005440°	6.665511°
D06	Le Cachot (NE)	Lawn with *Betula nana*	07/10/14	*Sphagnum capillifolium* & *S. magellanicum*	47.005738°	6.663991°
D07	Neuchâtel (NE)	Botanical Garden	11/10/14	Lower pool, with *Lemna*	46.999833°	6.934687°
D08	Neuchâtel (NE)	Botanical Garden	11/10/14	Lake, macrophytes with *Asellus*	46.999825°	6.936297°
D09	Neuchâtel (NE)	Botanical Garden	11/10/14	Higher pool, detritus	46.999959°	6.934659°
D10	Etang de la Gruère (JU)	Lake shore	13/10/14	Floating vegetation on shore: *Sphagnum cf. fallax*	47.238765°	7.052270°
D11	Etang de la Gruère (JU)	Lake shore opposite to the "peninsula"	13/10/14	Submerged vegetation	47.237978°	7.048248°
D12	Chaux des Breuleux (JU)	Floating vegetation around a large pond	13/10/14	wet *Sphagnum cf. fallax*	47.226242°	7.045749°
D13	Les Pontins (BE)	Small pool at the base of a peat slope	13/10/14	wet *Sphagnum cf. fallax*	47.127285°	6.989863°

DNA-based identification, using (1) a taxonomically informed species assignment with a reference database, and (2) an unsupervised assignment based on barcoding thresholds only. The main aim of the tests was to assess if we are now ready for such kind of inventories of biological diversity based directly on DNA and not only through morphological identification.

MATERIAL AND METHODS

Sampling

Authorisations were requested and obtained from local governments to sample *Sphagnum* mosses from selected peatlands in Switzerland, in the Jura Mountains (cantons of Neuchâtel, Jura and Bern) in relation to this project. Sampling took place mostly in October 2014. We collected samples in L'Etang de la Gruère (Jura/Bern), La Chaux-des-Breuleux (Bern/Jura), Les Pontins (Bern), Le Cachot (Neuchâtel) and Le Bois-des-Lattes (Neuchâtel) (Table 1). Each sample consisted in a cube of *Sphagnum* moss of 5 cm side, stored in a plastic bottle, and kept refrigerated in the field and during the transport to the laboratory.

Species identification

Animals were sorted and isolated in the laboratory under a dissecting microscope, taking as a representative subsample a cube of 1cm side from each sample. All isolated individuals were identified to species level or to genus level. Pictures were taken at a compound microscope at 200 to 400x magnification for each isolated individual. The identification characters for bdelloids are only visible on active individuals (Donner, 1965) and it is therefore impossible to fix the animals in such a way that characters are visible on a permanent slide, whereas they can still be visible on photographs.

DNA sequence data

DNA was extracted from single identified and photographed individuals of bdelloid rotifers using a Chelex extraction protocol (Gómez *et al.*, 2002). For each individual, partial COI mtDNA gene was sequenced adapting the protocol for monogonont rotifers (Gómez *et al.*, 2002): DNA from each single animal was extracted in 35 µL of Chelex (InstaGene Matrix; Bio-Rad, CA, USA). A 658 base pairs fragment of the COI gene was PCR amplified using optimized primers LCOI (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCOI (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') (Folmer *et al.*, 1994). Cycle conditions comprised initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 43 °C for 1 min and 72 °C for 90 s, and a final extension step of 72 °C for 7 min. Purification and sequencing were performed by an external company. Chromatograms were checked for ambiguous positions using FINCHTV 1.4.0, aligned with MAFFT (Katoh & Standley, 2013) with the default automatic settings, and visually checked by eye for correct protein coding in Mesquite (Maddison & Maddison, 2018).

Analyses

The first test was performed on the DNA barcoding pipeline that would be used to try to identify an organism starting from its DNA sequence and comparing it to a reference database. We performed this step by checking the highest similarity in the known DNA sequences available in the GenBank database (i.e. "best match") through BLAST searches (Benson *et al.*, 2013). For each of the sequences we obtained from the animals collected in the field, we gathered information on the GenBank best match regarding species identification, percentage similarity, and the length in base pairs of the overlapping part of the sequences.

We then checked how many of the retrieved best matches correctly identified the sequence to species and genus level, in accordance with our morphological identification. Ideally, the match should be 100%; yet, biological diversity is much higher than what we can actually describe, and some level of uncertainty is always expected. For example, it could be that different morphological species match to the same species name in the GenBank reference database, or that different individuals of the same morphospecies match to

Table 2. List of the 17 morphologically identified species of bdelloid rotifers from *Sphagnum* peatlands in the Swiss Jura Mountains, number of sequenced individuals and from how many populations they come, number of available COI sequences in GenBank for each species, number of successfully identified individuals from BLAST searches at the species, genus, and family level. In addition, the number of ABGD units for each species is provided in the last column. The last row summarises the totals for each column. *Lista de las 17 especies de rotíferos bdelloideos identificados morfológicamente de las turberas de* Sphagnum *de las montañas del Jura suizo, número de individuos secuenciados y de cuántas poblaciones provienen, número de secuencias de COI disponibles en GenBank para cada especie, número de individuos identificados con éxito usando BLAST a niveles de especie, género y familia. Asimismo, se proporciona el número de unidades ABGD para cada especie en la última columna. La última fila contiene las sumas de cada columna.*

species	individuals	populations	GenBank sequences	correct species	correct genus	correct family	ABGD units
Adineta gracilis Janson, 1893	8	5	26	8	8	8	1
Adineta steineri Bartos, 1951	1	1	13	1	1	1	1
Adineta vaga (Davis, 1873)	1	1	230	1	1	1	1
Dissotrocha aculeata (Ehrenberg, 1832)	2	1	23	2	2	2	1
Dissotrocha macrostyla (Ehrenberg, 1838)	14	4	26	3	13	14	3
Habrotrocha lata (Bryce, 1892)	3	2	2	2	2	2	2
Macrotrachela plicata (Bryce, 1892)	2	2	3	0	1	2	1
Macrotrachela quadricornifera Milne, 1886	7	3	102	7	7	7	2
Otostephanos donneri Bartos, 1959	1	1	4	0	0	0	1
Philodina citrina Ehrenberg, 1832	7	3	56	7	7	7	2
Philodina megalotrocha Ehrenberg, 1832	1	1	26	1	1	1	1
Philodina sp.	1	1	NA	NA	1	1	1
Rotaria macroceros (Gosse, 1851)	2	1	2	0	1	1	2
Rotaria magnacalcarata (Parsons, 1892)	4	1	56	4	4	4	1
Rotaria rotatoria (Pallas, 1766)	48	6	857	48	48	48	8
Rotaria sp.	6	4	NA	NA	6	6	2
Rotaria tardigrada (Ehrenberg, 1832)	8	4	15	8	8	8	1
total	116	13	1441	92	111	113	31

different species names. Such mismatches could be due to actual taxonomical uncertainties in the identification of cryptic or pseudocryptic species based on morphology, or to potential errors in the reference database; moreover, it could also be that the closest match in the reference database is still not so genetically similar and thus provides an unreliable and false best match.

To address these questions by assessing the type of errors that produced wrong taxonomic assignments through BLAST searches and including also other potential confounding factors, we performed statistical analyses through analyses of variance (ANOVA). We tested whether the percentage of similarity, or the length of the sequence, was different between correctly and incorrectly identified sequences. Moreover, by using species-level summary data (Table 2), we tested whether the proportion of correctly identified sequences for each morphological species could be due to the number of individuals for each species, as a proxy for sampling bias in the data, or to the number of sequences available in GenBank, as a proxy for bias in the reference database. We addressed this issue by using generalised linear models (GLM) with binomial error for proportion data (Crawley, 2012).

A different pipeline could be followed to describe diversity without any previous knowledge on DNA sequences on bdelloid rotifers, adopting an uninformed approach in the delimitation of species. Confirming the reliability of this approach would suggest that biodiversity analyses through DNA sequence data could be performed even in the absence of a reference database (Leese *et al.*, 2018). The use of DNA sequence data in the DNA taxonomy of understudied taxa is quite developed, with several methods that have already been applied to microscopic animals. Among these methods, we selected the Automated Barcode Gap Discovery, ABGD (Puillandre *et al.*, 2012), which is known to be reliable in COI of rotifers (Mills *et al.*, 2017) using the default settings of Pmin and Pmax on uncorrected genetic distances. Our dataset includes several individuals from few morphological species, limiting the problems in using ABGD with incompletely sampled taxa (Ahrens *et al.*, 2016). The ABGD approach identifies the best delineation of taxonomic units

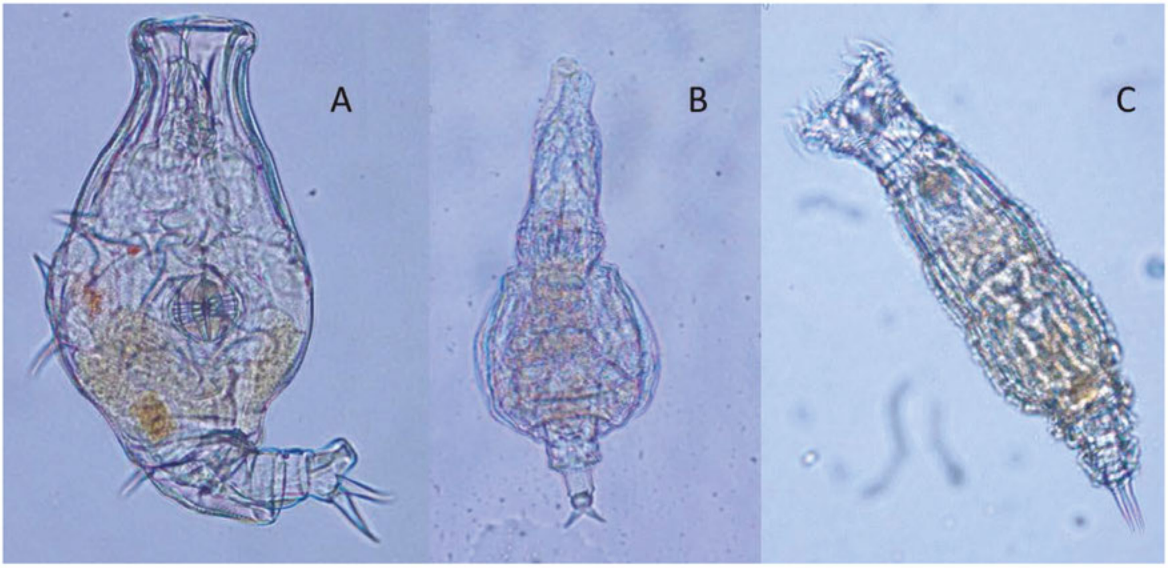

Figure 1. Examples of voucher photographs of the identified individuals of bdelloid rotifers that were processed for DNA extraction. A: *Dissotrocha aculeata* D10_DA01a; B: *Habrotrocha lata* D01_HL01c; C: *Dissotrocha macrostyla* D01_DM03a. *Ejemplos de fotografías de especímenes de referencia de los individuos identificados de rotíferos bdelloideos que se usaron para extracción de ADN. A:* Dissotrocha aculeata *D10_DA01a; B:* Habrotrocha lata *D01_HL01c; C:* Dissotrocha macrostyla *D01_DM03a.*

potentially equivalent to species on the basis of the clearest barcoding gap between them and it is unlinked to the availability of previous taxonomic knowledge. We assessed whether the units of diversity discovered by ABGD matched the morphological species or not. We then asked whether the number of ABGD units for each morphological species could be due to the number of individuals or of populations for each species. We addressed this issue by using generalised linear models (GLM) with quasipoisson error for count data (Crawley, 2012).

As a description of the genetic variability in bdelloid rotifers from *Sphagnum* bogs, we provided metrics of uncorrected genetic distances within and between taxonomic units. All analyses were performed in R 3.3.3 (R Core Team, 2017), with the package ape v5.0 for handling DNA sequence data (Paradis *et al.*, 2004).

RESULTS

In total, 17 morphological species of bdelloid rotifers were identified from the 13 samples (Fig. 1, Table 2), and 116 COI sequences were obtained (GenBank accession numbers MH251750-MH251865; Table S1, see Supplementary information, available at http://www.limnetica.net/en/limnetica). Out of the 109 animals identified to species level, 92 provided a correct species identification with BLAST searches against GenBank: thus, in 84.5 % of the cases, the highest match indeed identified the same name of the morphological species. The cases of mismatch referred to five morphological species, namely (see Supplementary information, Table S1).

For *Dissotrocha macrostyla*, most of the animals, 11 out of 14 (78.6 %), had the highest similarity to sequences of another species of the same genus, *D. aculeata* (see Supplementary information, Table S1). The genetic distance to the closest match was significantly smaller for the correctly identified animals (0.6 to 13.4 %) than for the incorrectly identified ones (13.2 to 14.3 %) (ANOVA: $F_{1,12} = 6.0$, $p = 0.03$).

For *Habrotrocha lata*, one animal out of three was not correctly assigned and had the closest match to a species of another family (*Pleuretra lineata*, family Philodinidae: see Supplementary information, Table S1). The correctly identified sequences had the closest match at genetic distances of 6.5 and 7.5 %, whereas the incorrectly identified one was at higher distance, 11.3 %.

For *Macrotrachela plicata*, none of the two animals was correctly assigned. The closest match corresponded to other species of the same family (see Supplementary information, Table S1), with genetic distances of 10.2 and 10.7 %.

For *Otostephanos donneri*, the only animal was incorrectly assigned to a species of a different family (see Supplementary information, Table S1), with a distance of 12.4 %.

For *Rotaria macroceros*, none of the two animals was correctly assigned. One was assigned to *R. rotatoria*, a species of the same genus (see Supplementary information, Table S1), with a distance of 10.8 % and the other to a species of a different family, with a distance of 12.1 %.

All other species were correctly assigned (see Supplementary information, Table S1), even the ones with several animals from different populations (Table 2). Overall, the genetic distance to the closest match was significantly smaller for the correctly identified animals (average: 5.2 %, range: 0.002 to 15.3 %) than for the incorrectly identified ones (12.9 %, 10.2 to 14.3 %) ($F_{1,114} = 44.3$, $p < 0.0001$). The length of the overlapping part of the sequences with the ones in GenBank was not significantly different between correctly and incorrectly identified animals ($F_{1,114} = 0.1$, $p = 0.92$). The proportion of identified individuals for each species was not affected by any of the included variables: neither by the number of animals sequenced for each species (GLM: $z = -0.1$, $p = 0.90$), nor by the number of sequences available in GenBank ($z = 0.0$, $p = 0.13$).

Using a taxonomically blind approach without the use of a reference database, the 116 COI sequences provided evidence of 31 ABGD taxonomic units from the 17 morphological species (Table 2): the barcoding gap identified in the whole dataset by the application of ABGD was between 3.0 % (maximum intra-unit genetic distance) and 8.0 % (minimum inter-unit genetic distance) (Table 3). While a barcoding gap existed between the 31 ABGD taxonomic units, no clear barcoding gap was visible between the 17 morphological species. For them, the minimum

Table 3. Intraspecific minimum, maximum and mean uncorrected genetic distances between the 31 ABGD taxonomic units, with sample size (N). Intraspecific values are not reported for units with only 1 individual and NA means that no distance could be calculated between animals because they had the same COI sequence. The maximum genetic distance within each ABGD unit was 0.030, on average 0.011. The distances between all ABGD units were minimum = 0.080, maximum = 0.242, mean = 0.161. *Distancias genéticas sin corregir intraespecíficas mínimas, máximas y medianas entre las 31 unidades taxonómicas ABGD, con el número de especímenes N. Los valores intraespecíficos no se calcularon para unidades con un solo individuo. NA significa que la distancia no se pudo calcular ya que todos los animales tenían la misma secuencia de COI. La distancia genética máxima entre cada unidad ABGD era de 0.030, con un promedio de 0.011. Las distancias entre todas las unidades ABGD eran de mínimo 0.080, máximo 0.242 y una media de 0.161.*

ABGD unit	minimum	maximum	mean	N
s02	0.002	0.008	0.004	11
s03	NA	NA	NA	3
s05	0.005	0.028	0.015	11
s06	0.005	0.030	0.017	8
s07	0.004	0.004	0.004	3
s08	NA	NA	NA	2
s09	0.002	0.024	0.008	18
s10	0.002	0.010	0.007	3
s12	0.002	0.025	0.015	8
s13	0.006	0.006	0.006	2
s14	0.006	0.006	0.006	2
s16	0.003	0.025	0.012	6
s19	0.002	0.003	0.002	4
s20	0.003	0.003	0.003	3
s21	0.002	0.002	0.002	3
s23	NA	NA	NA	2
s26	0.022	0.022	0.022	2
s30	0.016	0.029	0.021	9
s31	0.003	0.012	0.007	4

inter-specific genetic distance was still 8.0 % but the maximum intra-specific genetic distance was much higher (Table 4): 10.4 % for *M. quadricornifera*, 11.5 % for *P. citrina*, 12.8 % for *H. lata*, 13.5 % for *Rotaria* sp., 13.8 % for *R. macroceros*, 15.6 % for *D. macrostyla*, and even 19.5 % for *R. rotatoria*. The intraspecific value of 19.5 % for *R. rotatoria* is only slightly smaller than the maximum difference in the whole dataset of 116 sequences for all the bdelloids, 24.2 %.

Most of the morphological species with several individuals were split into several ABGD taxonomic units: the highest number was eight for *R. rotatoria*, represented by 48 animals, and three for *D. macrostyla*, represented by 14 animals (Table 2). The number of ABGD taxonomic units for each morphological species was strongly biased by the number of sequences for each morphological species (GLM: z = 4.9, *p* = 0.0002) but not by the number of different populations for each morphological species (z = 0.3, *p* = 0.77). Indeed, even though different ABGD units within the same morphological species were often found in different samples, these occurred also in a few cases in the same population. The most extreme case is that of the only two individuals of *R. macroceros* found in sample D10, which belonged to two different ABGD taxonomic units, s27 and s28 (Table 2) with a genetic distance of 13.8 % between them. The other instance of co-occurring ABGD taxonomic units within the same morphological species was for *R. rotatoria*, with two ABGD taxonomic units found in sample D03 (s09 and s11, 8.3-8.7 % distance between them), and even four ABGD units in sample D08 (s20, s21, s22, and s23, with 11.6-19.5 % distance between them).

DISCUSSION

The main result of our DNA barcoding survey of bdelloid rotifers from *Sphagnum* bogs in the Swiss Jura Mountains is that the approach provides rather consistent estimates of diversity

Table 4. Intraspecific minimum, maximum and mean uncorrected genetic distances between the 17 morphological species, with sample size (N). Intraspecific values are not reported for species with only one individual. In addition, the number of ABGD taxonomic units is reported for each species. The maximum genetic distance within each species was 0.195, on average 0.109. The distances between species were minimum = 0.080, maximum = 0.242, mean = 0.164. *Distancias genéticas sin corregir mínimas, máximas y media entre las 17 especies morfológicas, con número de individuos (N). Los valores intraespecíficos no se registraron para especies con un solo individuo. Asimismo, se reportó el número de unidades taxonómicas ABGD para cada especie. La distancia genética máxima dentro de cada especie era de 0.195, con un promedio de 0.109. Las distancias entre todas las especies eran de mínimo 0.080, máximo 0.242 y una media de 0.164.*

species	minimum	maximum	mean	N	ABGD
Adineta gracilis	0.002	0.025	0.015	8	1
Dissotrocha aculeata	0.022	0.022	0.022	2	1
Dissotrocha macrostyla	0.002	0.156	0.068	14	3
Habrotrocha lata	0.006	0.128	0.082	3	2
Macrotrachela plicata	0.006	0.006	0.006	2	1
Macrotrachela quadricornifera	0.003	0.105	0.068	7	2
Philodina citrina	0.003	0.115	0.038	7	2
Rotaria macroceros	0.138	0.138	0.138	2	2
Rotaria magnacalcarata	0.002	0.003	0.002	4	1
Rotaria rotatoria	0.002	0.195	0.120	48	8
Rotaria sp.	0.004	0.135	0.087	6	2
Rotaria tardigrada	0.005	0.030	0.017	8	1

with the traditional morphological methods. Overall, most of the DNA sequences of COI from the sequenced animals matched to the correct species name in the GenBank reference database. The 17 sequences (15.6 %) that did not match the correct species names corresponded to sequences that did not have any close match in GenBank and were thus incorrectly assigned: all of these incorrectly assigned animals had a genetic distance to the closest match above 10 %, which is a rather high genetic distance in COI for animals, even for rotifers (Tang *et al.*, 2014). A COI distance of 10 % is indeed typically above the barcoding gap used to separate two closely related species in many groups of animals (Hebert *et al.*, 2003).

In rotifers, it is known that animals of the same morphospecies may exceed 10 % in their genetic distances in COI (Fontaneto, 2014). Such high genetic distances within the same morphospecies is known in rotifers for cases of cryptic or pseudocryptic species (e.g. Mills *et al.*, 2017; Moreno *et al.*, 2017). Indeed, the use of a statistical approach to identify taxonomic units from DNA sequence data, such as the ABGD method we applied, revealed that several cryptic taxa could be potentially present in our dataset. Out of 17 morphological species, 31 ABGD units were found, with minimum genetic distances between them of 8 %. Such a threshold is lower than the 10 % distance of the incorrectly assigned sequences. Thus, we can support the hypothesis that all the misidentification we had were due to the occurrence of cryptic species coupled with the lack of corresponding DNA sequence information in GenBank for each cryptic species within the complexes.

We are confident that further studies would be able to fill this knowledge gap in the reference database: one of the extreme cases of the occurrence of cryptic species in bdelloid rotifers is *Rotaria rotatoria*, with an estimated number of few tens of species in the complex (Fontaneto *et al.*, 2009). Yet, because of a good representativeness of the species complex in the reference database, with more than 800 sequences already available in GenBank, none of the 48 animals of this morphospecies in our dataset was incorrectly identified. More taxonomic work should be performed for the morphospecies *R. rotatoria* in order to resolve the taxonomic ambiguity, following what was done for the emblematic case of the *Brachionus plicatilis* species complex (Mills *et al.*, 2017) for which 15 species were determined from integrative taxonomic approaches combining extensive barcoding, morphology, and also geographic information. Yet, notwithstanding the high taxonomic uncertainties for *R. rotatoria*, the survey we performed based on DNA sequence assigned all animals to the correct species complex, and the new genetic information provided by our survey further increased the reference database.

The lack of corresponding sequences in GenBank can be filled only by further faunistic and taxonomic studies including DNA sequence information, similar to the one we presented here. The need for a reliable reference database is one of the optimal requirements for biological monitoring of aquatic habitats through DNA barcoding and metabarcoding (Leese *et al.*, 2018). The endeavour of obtaining and managing such a database started several years ago with shared information through GenBank and BOLD, and for some groups it developed in taxonomically curated and reliable systems to query the sequences obtained from the field, for example in prokaryotes (SILVA, Quast *et al.*, 2013), in protists (UniEuk, Berney *et al.*, 2017), and in fungi (UNITE, Abarenkov *et al.*, 2010). Any metabarcoding study on protists sequenced from the field in bulk extractions of organisms or from environmental DNA has the very useful UniEuk system as a reference for protist species, but the same study will recover sequences from rotifers and from other microscopic animals such as nematodes, tardigrades, and gastrotrichs, which are of similar size as several protists and live in the same habitats. Thus, it would be useful to start a curated reference system also for microscopic animals, or even for them to be included in the UniEuk reference database for unicellular eukaryotes.

Overall, we can conclude that we are ready to assign bdelloid species identification starting from DNA sequence information in aquatic habitats. We were successful in spite of the fact that we focused on a previously understudied country and habitat for rotifers. Our suggestion for future applications is to adopt a confident approach and trust only close matches that are lower than 10 % in genetic distances, while leaving as unidentified all sequences that have a higher genetic distance. We are confident that in the future the representativeness of GenBank, BOLD, or any other dedicated reference system will improve, but at least for acidic aquatic habitats of Central Europe we demonstrated that the approach could be considered doable and reliable already now.

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Who is *Moina micrura*? Redescription of one of the most confusing cladocerans from *terra typica*, based on integrative taxonomy

Manuel Elías-Gutiérrez[1,*], Petr Jan Juračka[2], Lucía Montoliu-Elena[1,3], Maria Rosa Miracle[†], Adam Petrusek[2] and Vladimír Kořínek[2]

[1] El Colegio de la Frontera Sur, Unidad Chetumal, Av. Centenario Km 5.5, Chetumal 77014, Quintana Roo, México.
[2] Charles University, Faculty of Science, Department of Ecology, Viničná 7, Prague 2, Czech Republic.
[3] Posgrado en Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México; Av. Ciudad Universitaria 3000, ZIP. 04510, Coyoacán, Ciudad de México, México.
[†] University of Valencia (deceased).

[*] Corresponding author: melias@ecosur.mx

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ABSTRACT

Who is *Moina micrura*? Redescription of one of the most confusing cladocerans from *terra typica*, based on integrative taxonomy

Moina micrura Kurz, 1875 (Anomopoda: Moinidae) belongs among the most poorly defined cladoceran species in the world. This species has been considered cosmopolitan and is widely used for laboratory experiments, ecotoxicology, physiology or as live food. Nevertheless, recent molecular analyses corroborated the idea that it is a diverse complex of closely related species. Persisting systematic problems with *M. micrura* stem from the fact that it has not been redescribed in detail, and its type material as well as the type locality are lost. With this study, we try to provide a redescription, using morphology of females, males and ephippial females and the DNA barcodes for *M. micrura* s. str. from the pond situated not far from its original type locality in the Czech Republic. Firstly, we sequenced mitochondrial genes for cytochrome c oxidase subunit I (COI) and the 12S rDNA to establish the identity of *M. micrura* s. str. After this analysis, we compared the genetic data with all available sequences across the world. The species seems restricted to the Western Palearctic, with the most easterly located genetically confirmed records from Kazakhstan and Israel. The closest related lineage to *M. micrura* s. str. was found in Spain and needs a thorough analysis to establish its systematic status. *M. micrura* s. str. has unique features in the posterior spinulation of the valves of the parthenogenetic female, and the ornamentation of the ephippium. In the male, spinules on the tip of the antennule and the hook on the first thoracopod are also unique. With this description, we hope to inspire specialists to start uncovering the diversity of this complex, to understand its biogeography and diversity, as well as the real range of *M. micrura* s. str.

Key words: Cladocera, systematics, taxonomy, species complex, Western Palaearctic

RESUMEN

¿Quién es* Moina micrura*? Redescripción de uno de los cladóceros más confusos a partir de su* terra typica*, basada en la taxonomía integrativa

Moina micrura *Kurz, 1875 (Anomopoda: Moinidae) es una de las especies de cladóceros más confusas del mundo. Durante mucho tiempo fue considerada cosmopolita pero análisis moleculares recientes corroboraron la idea de que es un complejo de especies crípticas estrechamente relacionadas. Los persistentes problemas sistemáticos con* M. micrura *derivan de la falta de una redescripción detallada y de que tanto su material tipo como la localidad tipo han desaparecido. La importancia de esta especie radica en que es utilizada en diversos experimentos de índole ecotoxicológica, fisiológica, como alimento vivo, etc. Con este estudio, tratamos de proporcionar una redescripción de* M. micrura *s. str, utilizando la morfología de las hembras partenogenéticas, efipiales y de los machos, así como caracteres adicionales como los códigos de barras de ADN. El material* *estudiado procede de un estanque situado cerca de la localidad tipo original en la República Checa. En primer lugar, se secuenciaron los genes mitocondriales citocromo c oxidasa subunidad I (COI o códigos de barras) y el 12S a fin de establecer la identidad de* M. micrura *s. str. Después de este análisis, se compararon las secuencias obtenidas con todas las secuencias publicadas disponibles de esta especie a nivel mundial. Aparentemente,* M. micrura *s. str. está restringida al Paleártico, con registros confirmados molecularmente en República Checa, Eslovaquia, Kazajstán e Israel. El linaje más próximo a* M. micrura *s. str. se encontró en España pero se necesita un análisis más exhaustivo para establecer con certeza su identidad.* M. micrura *s. str. presenta características únicas como la espinulación posterior de las valvas de la hembra, y la ornamentación del efipio. En el macho, las espínulas en la punta de la antena y el gancho del primer toracópodo también son únicos. Con esta descripción, esperamos que los especialistas tengan una referencia clara para descubrir la diversidad de este complejo, así como comprender su biogeografía y diversidad.*

Palabras clave: *Cladocera, sistemática, taxonomía, complejo de especies, Paleártico occidental*

INTRODUCTION

Moina micrura, seemingly one of the most ubiquitous cladocerans with apparently worldwide distribution, has been also one of the most taxonomically confused species. It was described by Kurz (1875) from Bohemia (the present-day Czech Republic), in short paragraphs, where no presently relevant taxonomic characters were used. Although the description and accompanying drawings (Fig. 1) were exceptionally good for those times, they lacked fundamental diagnostic characters, and no characters of males or ephippial females were mentioned. Furthermore, the type material is lost and the original *locus typicus*, a fish pond Mlýnský (Muehlteich) at Malešov village, has been dried out decades ago and the surrounding area forested (Petrusek, 2002). The first attempt to review contemporary knowledge on *M. micrura* in Bohemia, its *terra typica*, was published by Šrámek-Hušek (1940), as the validity of Kurz' species was doubted (e.g., Wagler, 1937). Šrámek-Hušek (1940) presented a substantial amount of evidence to support the distinct status of the species, based on the ample material collected in the vicinity of the original type locality.

Later, due to the apparent intraspecific variability within *M. micrura*, at least three subspecies were described and accepted by specialists. Goulden (1968) in his classic monograph on the systematics of the Moinidae family included seven formerly separate species as younger synonyms of *M. micrura*, and later, two more were synonymized by Smirnov (1976). Since then, a great confusion has been apparent, and regional faunal books display *M. micrura* descriptions and drawings that do not match each other. For example, Alonso (1996) used Spanish material to illustrate *M. micrura*, but it differs in details with

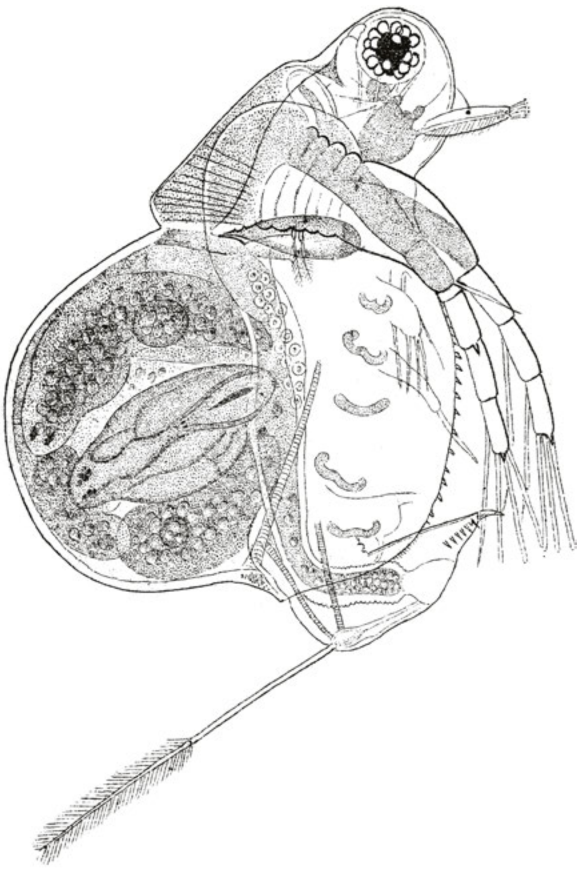

Figure 1. *Moina micrura* drawn by Kurz (1875) in the original species description. *Dibujo de* Moina micrura *realizado por Kurz (1875) en la descripción original de la especie.*

material from other regions (pers. obs.).

Hudec's publications (1990; 2010) contain a very good morphological characterization and figures of *M. micrura* s. str. based on Central European material from the Danube basin, and also provides diagnostic characters to distinguish it from a superficially similar species also found in the region, *Moina weismanni* Ishikawa, 1896. However, Hudec's descriptions of *M. micrura* do not include all thoracopods and other important details of the shell, and no molecular data are provided.

M. micrura, presumably widely distributed in Europe, has been also recorded from all other continents except Antarctica. In Asia, it has been reported for example in Turkey (Bekleyen *et al.*, 2008), southern China (Li *et al.*, 2012), Malaysia (Idris, 1983) and India (Chatterjee *et al.*, 2013). The last-mentioned authors regarded *Moina dubia* Guerne & Richard, 1892 as a valid species distinct from *M. micrura*. In the American continent, Elías-Gutiérrez *et al.* (2008) compiled the records related to this taxon in Mexico and regarded *M. micrura* as a complex of species even within that single country. Particularly in Brazil, presumed *M. micrura* has been recorded in diverse environments, from freshwaters to a brackish hyper-eutrophic estuary (Paranhos *et al.*, 2013; Paranaguá *et al.*, 2005). It has also been reported from Australia (Smirnov *et al.*, 1983; but see Petrusek *et al.*, 2004) and from the whole African continent (Dumont *et al.*, 1981).

As detailed morphology of most branchiopods remains not satisfactorily described, it is considered that molecular data could help to understand diversity and phylogeny of this group (DeWaard *et al.*, 2006). In the case of *M. micrura*, as far as we know, at least three studies involved some molecular analyses, but they are partial and do not clarify the taxonomy of this species sufficiently. Petrusek *et al.* (2004) questioned the apparent cosmopolitanism of *M. micrura*, demonstrating by crossing experiments and molecular characters (sequence divergence of the mitochondrial gene for 12S rRNA) a distinctness of *M. micrura* from the Czech Republic (sampled about 90 km from the original type locality) from Australian *M. micrura*-like specimens. However, the genetic comparisons were based only on one clone of both species, and their morphology was not described. Elías-Gutiérrez *et al.* (2008) found in Mexican populations morphologically similar to *M. micrura* three related but distinct lineages based on the DNA barcodes, i.e., fragments of the cytochrome c oxidase subunit I gene, COI (Hebert *et al.*, 2003a, b). However, they could not compare these lineages with the European populations, because there were no sequences of *M. micrura* from terra typica available for this gene, which has become the standard marker for identification of animal species, including crustaceans (Eischeid *et al.*, 2016). A recent DNA barcoding-based study uncovered 21 phylogenetic groups of *Moina* within the Palearctic, with at least three clades of *micrura*-like taxa from this region (Bekker *et al.*, 2016).

Despite the taxonomical problems, many papers dealing with culture, ecotoxicology, ecology and other topics related to *M. micrura* have been published recently (58 papers are recorded in the main collection of the Web of Knowledge, Thomson Reuters, between 2010 and 2017), obviously due to a wide range and ubiquity of this taxon. However, many – possibly most – of these studies probably do not deal with *M. micrura* in a strict sense, and consequently results of such studies are not fully comparable.

The aim of our study is therefore to establish the identity of *Moina micrura* from its type locality, and to provide a good reference for future comparison and identification of this species. To achieve this goal, we used an integrative taxonomy approach (Dayrat, 2005), combining detailed morphological analyses and additional DNA sequencing for two genes. We also provide a short description of differential characters of *Moina weismanni* Ishikawa, 1896, as it often co-occurs in ponds with *M. micrura* within its *terra typica* (Petrusek, 2002), and their parthenogenetic females are superficially similar. Furthermore, we explored variation in ultrastructure of ephippia, as a potentially highly relevant taxonomic trait (Goulden, 1968; Juracka *et al.*, 2016) in populations of the *Moina micrura* complex across the globe, and compared them with several other species of the genus.

MATERIAL AND METHODS

Field Sampling

Material of *Moina micrura* was collected with a plankton (45 µm) and a hand net (90 µm) from a small fish pond named Sádka (49.963 N, 15.329 E; area 0.85 ha) situated between Ovčáry and Nové Dvory villages, 4.6 km east from Kutná Hora, in the Czech Republic, very close to the original (now dried up) type locality (Supplementary information, Table S1, available at http://www.limnetica.net/en/limnetica).

Material examined

All material (see Supplementary information, Tables S1 and S2, available at http://www.limnetica.net/en/limnetica) was preserved with non-denatured ethanol and since 2012, the procedure suggested by Prosser *et al.* (2013) was followed. Samples for intercontinental comparison are from V. Kořínek's collection of Cladocera (www.cladocera-collection.cz) and were originally preserved in 4 % formaldehyde solution and stored in 70 % ethanol.

As *Moina micrura* s. l. is widely distributed, we examined ephippial ultrastructure by scanning electron micrography on the material sampled from various continents, including Europe, Asia, Africa, South America, and Australia (see Supplementary information, Table S2) to evaluate morphological variation among populations isolated by large inter-continental distances. However, this variability within the species complex has to be compared also with the material from other well-defined species of the genus to evaluate the extent of interspecific variation. Therefore, we also included in the comparison *M. belli*, *M. brachiata*, *M. macrocopa*, *M. mongolica*, *M. reticulata*, *M. tenuicornis*, as well as morphologically clearly distinct but apparently undescribed lineage from Australia (Supplementary information, Table S2).

Analyses of the material

Morphological observations

Specimens were sorted from the ethanol-preserved samples under a stereomicroscope and placed in a drop of a glycerol. Several females were dissected. Whole animals and dissected sections were examined and measured under a differential interference contrast microscope and/or phase contrast microscope. They were morphologically identified following descriptions by several authors (Goulden, 1968; Alonso, 1996; Hudec, 2010).

Selected individuals were prepared for scanning electron microscopy (SEM). To remove unwanted biofilm covering surface of studied individuals, examined material was cleaned for 10 minutes with hot 10 % potassium hydroxide prior to its dehydration. Dehydration series followed standard graded series of acetone solutions in alcohol 30, 50, 70, 80, 90, 95 and 97 %, followed by two immersions in 100 % acetone. Afterwards, we replaced acetone with the hexamethyldisilazane for 20 minutes and left the material overnight in the desiccator. The dried samples were gold-coated and then observed with JEOL JSM-6380 LV scanning electron microscope at 15 kV. Further details of the methods are described in Juračka *et al.* (2016). Ephippial ultrastructure was always studied above the center of the egg chamber.

Selected molecular markers

Two mitochondrial genes previously sequenced in *M. micrura*-like animals (12S and COI) were selected as molecular markers also for this study. A fragment of the gene for 12S rRNA (343 bp) was selected to allow comparison with the previously published sequence of *M. micrura* s. str. by Petrusek *et al.* (2004), and the gene for COI (620 bp) due to its status as a standard barcoding gene in animal kingdom (Hebert *et al.*, 2003a; Eischeid *et al.*, 2016), its frequent use to discriminate cladoceran species (Elías-Gutiérrez *et al.*, 2008), and its availability for various *Moina* lineages (Elías-Gutiérrez *et al.*, 2008; Bekker *et al.*, 2016).

DNA isolation, PCR amplification and sequencing

DNA was extracted from whole-body homogenates using a mix of Proteinase K with invertebrate lysis buffer and digested overnight at 56 ºC for material processed before 2011 (see Elías-Gutiérrez *et al.*, 2008). Genomic DNA was subsequently extracted using a membrane-based approach. After 2011, we used the HotShot extraction protocol (Montero-Pau *et al.*, 2008). Approximately 600-658 bp were amplified for the COI using LCO1490 and HCO2198 primers (Folmer *et al.*, 1994) and/or ZPLK primers suggested by Prosser *et al.* (2013).

The polymerase chain reaction (PCR) reagents used were as follows: 12.5 µL of PCR reaction mix included 6.25 µl of 10 % trehalose, 2 µl of distilled deionized water and 1.25 µl of 10× PCR buffer for the enzyme Taq Platinum, 0.625 µl of $MgCl_2$ (50 mM), 0.125 µl of each primer, forward and reverse (0.01 mM), 0.0625 µl of dNTP mix (10 mM), 0.06 µl Platinum Taq polymerase (5 U/µl), and 2.0 µL of template DNA.

The thermocycler program included initial denaturation at 94 °C for 1 minute, 5 cycles of 94 °C for 40 seconds, 45 °C for 40 seconds, 72 °C for 1 minute, 35 cycles of 94 °C for 40 seconds, 51 °C for 40 seconds, 72 °C for 1 minute, and a final extension at 72 °C for 5 minutes.

For 12S, the PCR reaction mix was identical to that of COI but primers L13337-12S and H13842-12S were used (Machida *et al.*, 2004). The PCR was performed under following conditions: 95 ºC at 4 min, followed by 40 cycles of 94 ºC for 45 seconds, 60 ºC for 45 seconds, 72 ºC for 90 seconds, and the final extension step at 72 ºC for 6 minutes. PCR products were visualized on pre-cast agarose gels (E-Gels©, Invitrogen). They were then sequenced bidirectionally using an ABI 3730 (Applied Biosystems) capillary sequencer using the BigDye© Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), as described in Hajibabaei *et al.* (2005).

Sequence analysis

COI sequences obtained in this study (Supplementary information, Table S1) were combined with those for *M. micrura* and other members of this genus available in GenBank and Barcode of Life Database (BOLD, boldsystems.org) (Supplementary information, Table S1), to examine clade diversity. All sequences generated from this study are in the dataset DS-MICRSSTR *Moina micrura* s. str. description in BOLD and GenBank (see Supplementary information, Table S1 for accession numbers).

Alignments and calculations were made with the tools provided by BOLD (Ratnasingham *et al.*, 2007). COI sequences were aligned with BOLD aligner (Amino Acid based HMM) and 12S sequences were aligned with Kalign algorithm (Lassmann *et al.*, 2005). Nucleotide divergence was calculated using the Kimura two-parameter (K2P) algorithm (Kimura, 1980) with complete deletion of gaps and missing data. Neighbour-joining (NJ) trees (Saitou *et al.*, 1987) based on K2P distances (ID Tree) were created to provide a graphic representation of divergence patterns among species and lineages. For COI-based analyses, a sequence of *Moina macrocopa* from Texcoco Lake (Mexico) was used as outgroup; a sequence of *Moina macrocopa* from Kostelec (Czech Republic) was used for the same purpose when analysing 12S sequences (see Supplementary information, Table S1).

We also performed a maximum likelihood analysis with the MEGA6 software (Tamura *et al.*, 2013). A maximum likelihood tree was constructed using the General Time Reversible model with Gamma distributed and invariant sites (GTR G+I) as the best fitting model of substitution. Gamma distribution was approximated using five rate categories and nearest-neighbour interchange was used as heuristic method for tree inference. Nodal support for the resulting branches was estimated with 1000 bootstrap replications. The subtrees for terminal clades were collapsed for visualization in MEGA6.

Delimitation of groups within the *M. micrura* complex

Molecular operational taxonomic units (OTU's) have frequently been used to infer putative species boundaries where morphological identifications are difficult (Ashfaq *et al.*, 2015). We used two approaches to assign the 12S and COI sequences presumably belonging to the *M. micrura* species complex to OTU's: Barcode Index Number (BIN) system (Ratnasingham *et al.*, 2013) and Automatic Barcode Gap Discovery (ABGD; Puillandre *et al.*, 2012). The BIN system

Table 1. Genetic divergences (K2P) among the 12S (A) and COI (B) sequences for *M. micrura* s. l. All divergences are calculated as comparisons to *M. micrura* s. str. from the fishpond Sádka at Nové Dvory, the locality from which a neotype was selected. *Needs a revision to evaluate its distinctness or conspecifity with *M. micrura* s. str. **Only one sequence is available. *Divergencias genéticas (K2P) entre las secuencias de los genes 12s (A) y COI (B) para* M. micrura *s.l. Todas las divergencias están calculadas como comparaciones con* M. micrura *s. str. del estanque de peces Sádka en Nové Dvory, la localidad de donde fue selecciona el neotipo. *Necesita una revisión para evaluar su diferencia o co-especificidad con* M. micrura *s.str. **Solo una secuencia se encuentra disponible.*

(A)

Country	Locality (clade)	12S divergence
Czech Republic	Sádka	0
	Dobříň	0.19
	Ovčáry	0
Spain	Albufera lake (1 ES)	1.32*
	Sobron reservoir (1 ES)	1.32*
	Albufera lake (2 ES)	8.96
	Zahillo (3 ES)	11.31
Israel	Kinneret	0
Ethiopia	Dibdibo	6.46
Slovakia	Hrhov	0.19
Hungary	Czista Puszta	10.22
Australia	Albury	8.95

(B)

Country (lineage)	COI Divergence	
	Min Dist (%)	Max Dist (%)
Czech Republic	0**	0**
Kazakhstan	0.46	0.46
Spain (1 ES)	4.64	4.64
India	11.58	11.58
Spain (2 ES)	15.34	15.34
Russia	17.68	18.09
South Korea, Russia, Hungary	15.99	15.99
Mexico (MX 1)	15.24	16.37
Mexico (MX 2)	15.11	15.46
Mexico (MX 3)	13.28	14.30
Mexico (MX 4)	14.70	14.70
Mexico (MX 5)	13.52	13.52

is based in the Refined Single Linkage (RESL) algorithm to reach decisions on the number of OTU's in a sequence dataset through a three-phased analysis. ABGD employs a multi-phase system which initially divides sequences into OTUs based on a statistically inferred barcode gap (i.e., initial partitioning), and subsequently conducts additional rounds of splitting (i.e., recursive partitioning). The COI and 12S sequences were analyzed with an online version of ABGD (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) using K2P as the distance metric, employing a relative gap (X) of 1.5, a minimal intraspecific distance (Pmin) of 0.001, and a maximal intraspecific distance (Pmax) ranging from 0.02 to 0.1.

RESULTS

Sequence analysis

Kimura 2-parameter (K2P) distances among the 12S and COI sequences of putative *M. micrura* from various regions ranged from 0 to 11.3 % and from 0.27 to 18.09 %, respectively (Table 1, A and B). COI gene is less conservative than 12S, hence divergences are bigger. COI sequences with divergences exceeding 3 % were considered to belong to different groups (Hebert *et al.*, 2003b).

Analyzing the results of both genes and using the above-mentioned divergence threshold of 3 %, *Moina micrura* s. str. has been confirmed in the Czech Republic, Israel (Lake Kinneret; in this case using the 12S fragment, with 0 % divergence) and European part of Kazakhstan (near Ushkempir). The other groups represent nine divergent lineages, potentially cryptic species, of the *M. micrura* complex, the most closely related one originating from Spain.

Comparison of 12S and COI sequence variation

The results of the ID tree analysis in BOLD (Fig. S1, Available at http://www.limnetica.net/en/limnetica) and the maximum likelihood method (Figs. 2a, b and Fig. S2, http://www.limnetica.net/en/limnetica) were similar for both genes.

In the 12S tree (Figs. 2a, S2), the first group belongs to the species *M. micrura* s. str., it includes specimens from *terra typica* (Nové Dvory, CZ). The specimen sequenced by Petrusek *et al.* (2004) from a sandpit near Dobříň (CZ) (Supplementary information, Table S1) was grouped together with these, as well as specimens from Slovakia and Israel, in the same way as in the BOLD Id tree (Fig. S1). The closest relative to this clade is *M.* cf. *micrura* 1 ES from Spain (BIN: ACA1509). The second group forms a consistent cluster and is apparently restricted to Spain (ES). The two next groups which include sequences from Ethiopia (ET) and Australia (AU) are singletons, so further sequencing is needed to assess the distribution and variation within these groups. The fourth group is another species of the *micrura*-complex (*Moina* cf. *micrura* 2 ES), occurring in sympatry with *M.* cf. *micrura* 1 ES in the Albufera of Valencia lake (Spain). Next cluster groups together a sequence from Hungary (HU) and another from Zahillo pond in the Doñana National Park, South Spain (*M.* cf. *micrura* 3 ES).

The COI tree (Figs. 2b, S1), which allowed us to compare sequences from other parts of the world, included eleven groups that were recognized as distinct OTUs by both BIN and ABGD approaches. This wider comparison gave related results (comparable to those of Bekker *et al.*, 2016): the first group is *Moina micrura* s. str. and include sequences from the Czech Republic and Kazakhstan. The nearest group, as in 12S tree, is the cluster represented by the Spanish *Moina* cf. *micrura* 1 ES. The remaining nine groups are possibly distinct species, each with a geographically restricted distribution. The regions with *Moina micrura* s. str., confirmed by our results, are restricted to four: the Czech Republic, nearby Slovakia, Israel and the easternmost record in Kazakhstan.

Molecular analyses thus confirm that *Moina micrura* s. l. is a diverse group of species, with *M. micrura* s. str. apparently restricted only to a limited region of the Old World, probably western part of the Palearctic region only. However, before any nomenclatural act is taken to describe all these presumably new cryptic species or resurrect old names at present considered synonymous to *M. micrura*, it is necessary a complete an adequate morphological description of *Moina*

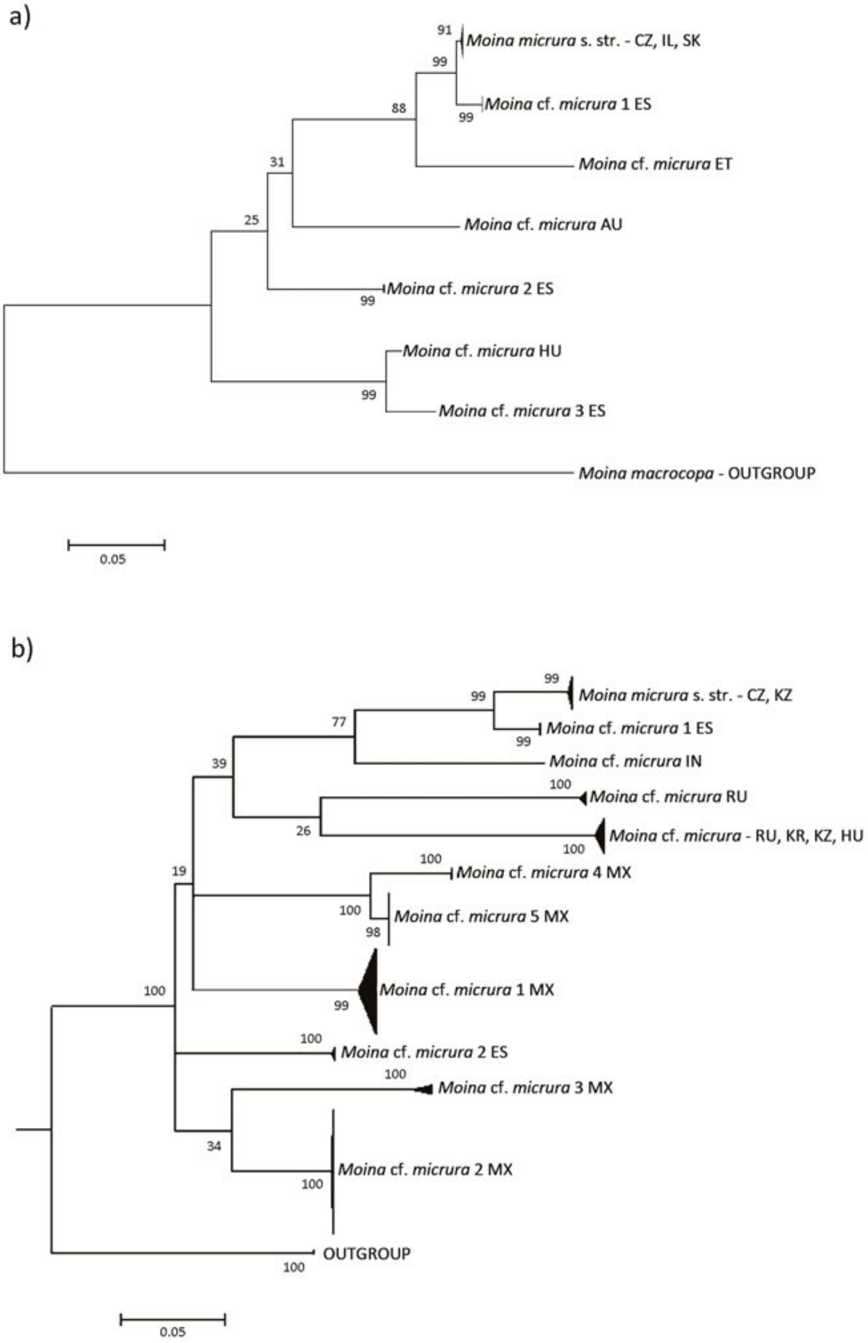

Figure 2. Maximum Likelihood trees of the *Moina micrura* complex based on analyzed mitochondrial genes. A. Tree based on 29 sequences of 12S gene; *Moina macrocopa* from the Czech Republic was used as an outgroup. B. Tree based on 148 COI sequences from the *M. micrura* complex; *M.* cf. *macrocopa* from Mexico was used as an outgroup. Bootstrap values (1000 replicates) are shown above the branches. The scale bar shows K2P distances. The node for each clade with multiple specimens is collapsed. The expanded tree is found as Fig. S4. *Árboles de máxima verosimilitud del complejo Moina micrura basado en los genes mitocondriales analizados. A. Árbol basado en 29 secuencias del gen 12S; se usó* Moina macrocopa *de la República Checa como grupo externo. B. Árbol basado en 148 secuencias del COI del complejo* M. micrura*;* M. *cf.* macrocopa *de México se usó como grupo externo. Se muestran los valores de bootstrap (1000 réplicas) por encima de cada rama. El valor de la barra de escala muestra las distancias K2P. El nodo para cada clado con múltiples especímenes está colapsado. El árbol expandido se encuentra en la Fig. S4.*

micrura s. str. Because of this reason, we provide in the next part a detailed description of *M. micrura* s. str. from *terra typica*.

Redescription of *Moina micrura* Kurz, 1875

Synonymy uncertain

Neotype: adult parthenogenetic female from Sádka pond stained with lignin pink and chlorazol black E; mounted in Canada balsam.

Type locality: Czech Republic, fish pond Sádka, at Nové Dvory village, NE of Kutná Hora, 49.963 N, 15.329 E. Repeated sampling from 2002 to 2015 & laboratory culture of specimens collected in the same pond (A. Petrusek & M. Miracle *legerunt*).

- Distribution of specimens from "*terra typica*" (watershed of the Elbe River in Central Europe) among museums:
- National Museum, Natural history collections, Prague:

1. One permanent mount of a *Moina micrura* Kurz, 1875 female – *neotype* specimen. Stained with a mixture of lignin pink & chlorazol black E and mounted in Canada balsam. Sádka Pond, Kutná Hora, Nové Dvory. 27.6.2002. A. Petrusek *legit*. Cat. Nr. P6E 4160
2. Four permanent mounts stained and mounted as above, specimens collected in the same locality. P6E 4161 & P6E 4162 (zooplankton sample), P6E 4163 (females) and P6E 4164 (males). Laboratory culture.
3. One permanent mount of *Moina micrura* from Upper Školní Pond, near Chabařovice. 8.8.2004. K. Pilařová *legit*; stained as above, mounted in Hydro-Matrix synthetic water miscible resin. P6E 4165.
4. One permanent mount of *Moina micrura* from a sand pit at Dobříň, NE of Roudnice nad Labem. 19.9.1999. A. Petrusek *legit*. Specimens cleared in hot lactic acid, stained as above, mounted in Canada balsam. P6E 4166.
5. One vial with zooplankton sample from the neotype locality, 27.6.2002, A. Petrusek *legit*. Preserved in 70 % ethanol. The species *Moina micrura* predominant, *Moina weismanni* Ishikawa, 1896 accessory. P6E 4169.

- Natural History Museum UK, London

1. One vial with zooplankton sample from the neotype locality, 27.6.2002. A. Petrusek *legit*. Preserved in 70 % ethanol. Cat. Nr. NHMUK 2017.38-47

Diagnosis

Parthenogenetic female

With round body, dorsum of valves elevated behind head when embryos present, convex or slightly straight in posterior part, no hairs on head and valves. Head with marked supraocular depression, no rostrum; ocellus without pigment. Denticles at posterior margin of valves organized in groups, each group with 7-10 elements increasing in size posteriorly. Postabdomen with row of 4-5 scale-like teeth fringed with row of marginal setae, and bident tooth with distal branch always significantly longer than proximal branch. Row of fine setae at the base of bident. Three groups of denticles and setules (pectens) on outer face of postabdominal claw; basal and middle pectens consisting of 4-5 and 8-12 strong thick spines, respectively, arranged fan-like; distal pecten with fine spinules. Basal and middle pectens often fused in continuous row. Antennule rod-like, with line of long setules on posterior face and sensory seta in middle, groups of minute spines with variable arrangements. Antenna with two coxal sensory setae, one short and other long reaching nearly to top of basal segment. Basal segment with minute distal spine on dorsal face, and remarkably long distal sensory seta on ventral face. Thoracopod I (Th1) with inner distal lobe (IDL) bearing a single strong anterior seta and 2 posterior soft setae. Endite 3 (= "penultimate segment") with anterior seta, endite 1 with three posterior setae and two ejector hooks, one large and other small.

Ephippial female

Ephippium with one egg. Free ephippium from lateral aspect broadly rounded anteriorly, posteriorly slightly tapering. Ephippium with robust dorsal ridge, its surface covered with hexagonal pattern.

Male

Body elongated. Dorsal margin nearly straight or feebly convex. Head small, wedge-like; filled up by optic vesicle. Ocellus without pigmentation. Ventral face slightly convex with antennule inserted laterally. Antennule long, apical sensory setae relatively long, two more setae (male seta and a sensillum) near first third of antennule. Four apical hook-like spines of different sizes. Trunk thoracopod I with a long, curved copulatory hook, its tip with two small claw-like projections and wart-like outgrow near inner base of its curvature. Large basal part of hook rounded and covered with dense mat of short hairs. Postabdomen with row of 4 plumose teeth and bident tooth, length of both branches of bident as in female. Row of fine setae at base of bident. Pectens on dorsal face of postabdominal claw as in female. Group of three oblique denticles near base of claw on the ventral side. Lateral gonopores situated on each side of postabdomen close to respective proximal-most plumose teeth.

Detailed description

Adult parthenogenetic female (Fig. 3A)

Length: 0.44-0.76 mm (n = 25). Body nearly rounded in lateral aspect, high (body height to length ratio 0.54-0.68), maximum height in middle-posterior portion, depending on number of embryos. Dorsum of valves strongly convex, projected much higher than head, almost straight in posterior part, dorsal depression between head and rest of body present. Postero-dorsal angle well-marked, rounded. Posterior margin more or less straight, continuing into widely convex ventral margin. No sculpture on surface of valves. No cuticular hairs on head and valves, without dorsal keel. Live animals are whitish in color.

Head – relatively large, rounded, without any rostral projection; with marked supra-ocular depression and large compound eye. Optical vesicle contiguous with prominent top of head. Ventral margin depressed under compound eye and bulging at level of antennules. Ocellus without visible pigmentation or with few grains of pigment (Fig. 3A).

Labrum – with fleshy main body, its ventral margin slightly convex.

1st maxilla – with three long strongly curved setae and one short stump-like outgrow, all with secondary setules.

Mandible – with asymmetrical, bifurcated anchoring projection.

Antennule (A1) – rod-like, about four times longer than wide, almost cylindrical, with transverse rows of scattered denticles on anterior face and row of long setules on posterior face (Fig. 3P). Antennular sensory seta conical, elongated, arising approximately in middle of lateral face. Distal tip bordered with small thick spinules. Nine short aesthetascs, two of them slightly longer. Tip of each aesthetasc with small projections giving an aspect of crown.

Antenna (A2) – similar in parthenogic and ephippial female. Coxal part with two setulated sensory setae, one long reaching up to ¾ of basal segment length, the other short and thin (Fig. 3N, arrows). Basal segment robust, with distal spine at dorsal face as long as first segment of 4-segmented antennal branch (Fig. 3O, magnification); long distal sensory seta on ventral face (Fig. 3O, arrow). Transverse rows of numerous, minute spinules on surface. Antennal branches elongated, 4-segmented exopodite slightly shorter than 3-segmented endopodite, all segments cylindrical, with rows of minute spinules and rows of long and thin setae. Antennal formula: setae 0-0-1-3/1-1-3, spines 0-1-0-1/1-0-1. Three long, apical swimming setae on both antennal branches, all with basal and distal segments bilaterally armed with fine, long setules. Lateral seta on third segment of dorsal branch with similar armature. Basal and distal lateral setae of ventral branch armed in another manner: basal segments unilaterally setulated and distal ones asymmetrically setulated. Spine on first endopodite segment short, like apical spines.

Valves – large, sub-ovoid, with row of 14 to16 setae along anterior ventral margin. Posterior part with row of marginal denticles, organized in groups of 7-10, increasing in size distally within each group. Denticles in posterior most margin form uniform row. Two posterior hooks supporting postabdominal setae (*setae natatoriae*) present.

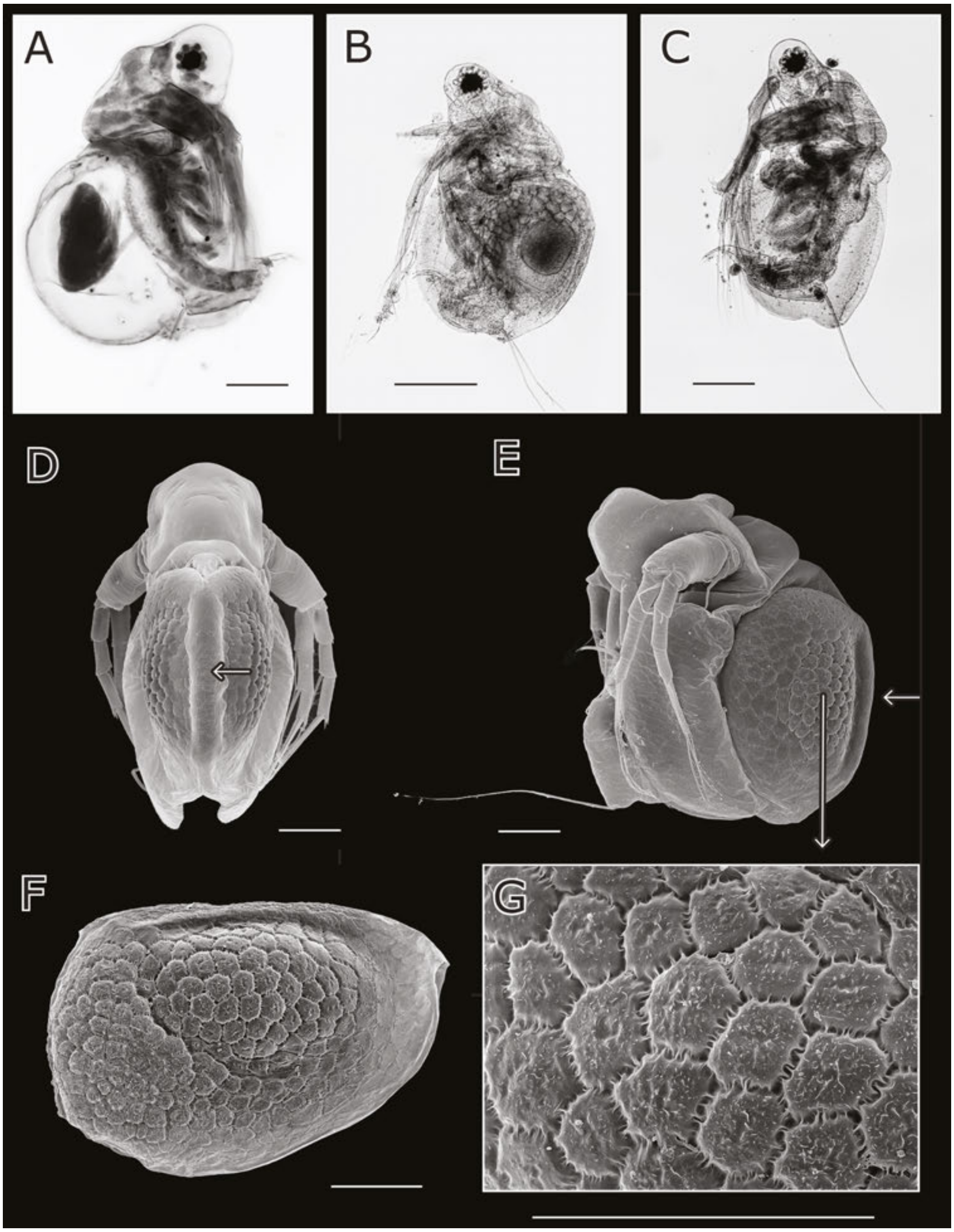

Figure 3. Morphological features of *Moina micrura* s. str. Specimens were sampled at three localities in the Czech Republic: fishpond Sádka at Nové Dvory (27.06.2002, images A, B, C, F, G, I, Q, R, S, T, U & V); fishpond Školní at Chabařovice (2.08.2004, images D, E, H, J, K, L, M, N & O), and flooded sandpit near Dobříň (5.10.1999, image P). A-C: light microscope, focus stacked images. A) Adult parthenogenetic female. B) Adult ephippial female. C) Adult male. D-R: SEM. D) Adult ephippial female in dorsal view, dorsal ridge indicated by arrow. E) Adult ephippial female in lateral view, dorsal ridge indicated by arrow. F) Free ephippium in lateral view. G) Ultrastructure of free ephippium. *Características morfológicas de* Moina micrura *s. str. Especímenes recolectados de tres localidades de la República Checa: el estanque de peces Sádka en Nové Dvory (27.06.2002, imágenes A, B, C, F, G, I, Q, R, S, T, U y V); estanque de peces Školní en Chabařovice (2.08.2004, imágenes D, E, H, J, K, L, M, N y O), y arenal inundado cerca de Dobřín (5.10.1999, imagen P). A-C: microscopio óptico, con las imágenes apiladas. A) Hembra partenogenética adulta. B) Hembra efipial adulta. C) Macho adulto. D-R: SEM. D) Hembra efipial adulta en la vista dorsal, cresta dorsal indicada por una flecha. E) Hembra efipial adulta en vista lateral, cresta dorsal indicada por una flecha. F) Efipio libre en vista lateral. G) Ultraestructura del efipio libre.*

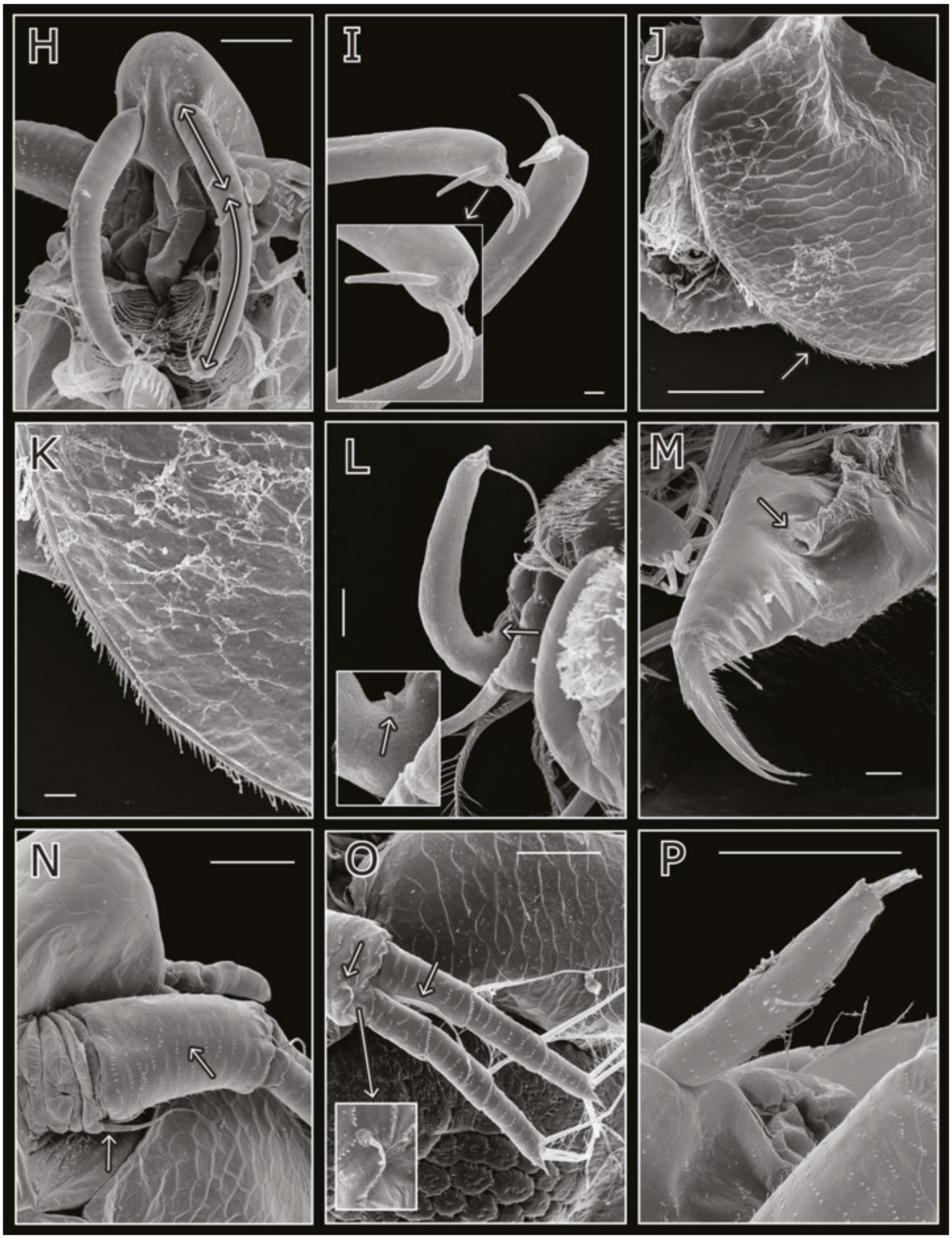

Figure 3 (cont.). H) Adult male, head in the ventral view. Arrows highlight proportion of proximal and distal antennular parts. I) Adult male, tips of antennules (detail). J) Adult male, ventral carapace margin. Arrow indicates spines along posterior ventral margin. K) Magnified detail of the spines along posterior ventral margin (as in previous image). L) Adult male, 1st thoracopod, arrows indicate small outgrow at base of the hook and its detail in the inset. M) Adult male postabdomen, arrow indicates gonopore. N) Ephippial female, basipodite of antenna. Arrows point to arrangement of minute spines on its surface and two sensory setae at its base. O) Ephippial female, antenna – arrows indicate the apical dorsal spine and ventral seta of the basipodite. P) Adult female, antennule. *H) Macho adulto, cabeza en vista ventral. Las flechas resaltan la proporción de partes antenulares proximales y distales. I) macho adulto, puntas de las anténulas (detalle). J) Macho adulto, margen del caparazón ventral. La flecha señala las espinas a lo largo del margen ventral posterior. K) Detalle de las espinas a lo largo del margen ventral posterior (como en la imagen anterior). L) Macho adulto, primer toracópodo. Las flechas indican una pequeña protuberancia en la base del gancho y su detalle en el recuadro. M) Macho adulto, postabdomen, la flecha indica el gonoporo. N) Hembra efipial, basipodito de la antena. Las flechas apuntan a la disposición de diminutas espinas en su superficie y dos setas sensoriales en su base. O) Hembra efipial, antena: las flechas indican la espina dorsal apical y la seta ventral del basipodito. P) Hembra adulta, primera anténula.*

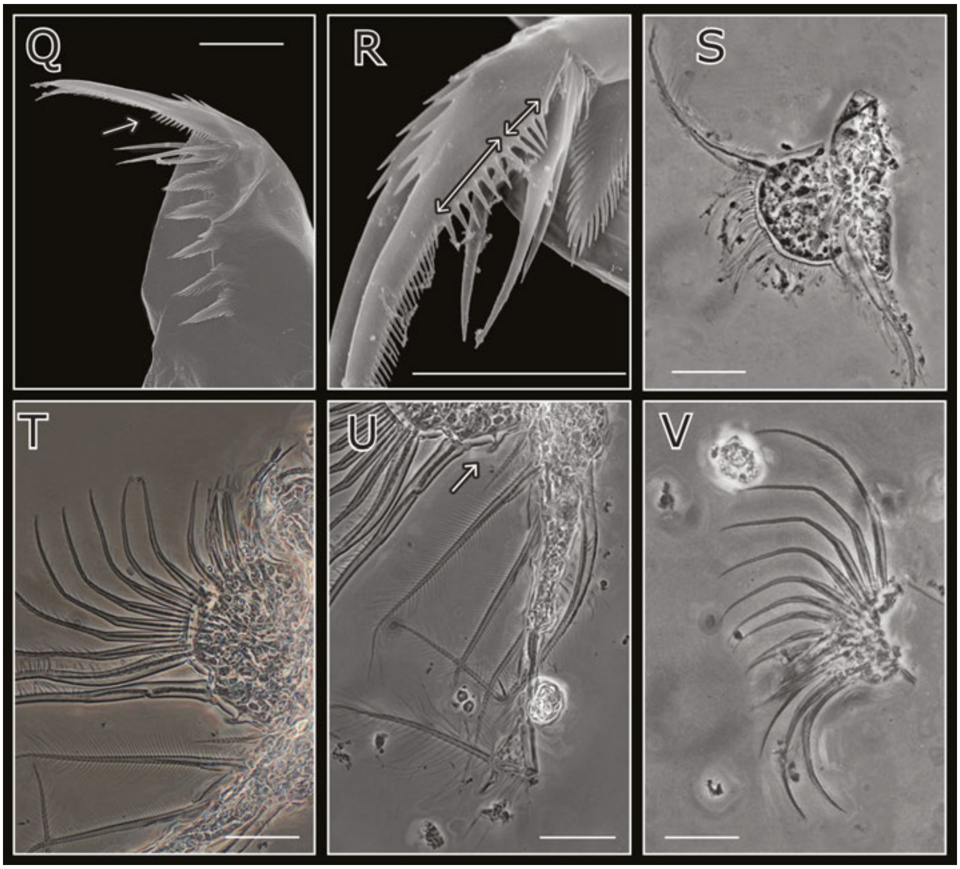

Figure 3 (cont.). Q) Adult female, postabdomen, arrow indicates the middle pecten. R) Adult female, base of postabdominal claw with three dorsal pectens (proximal and middle indicated by arrows) and group of robust ventral teeth. S) Adult female, 5th thoracopod. T) Adult female, 2nd thoracopod. U) Adult female, exopod in 2nd thoracopod. V) Adult female, 2nd thoracopod gnathobase. Scale bars indicate 0.1 mm (A-G, J), 10 µm (K-M, Q, R), 5 µm (H, N, P) and 25 µm (S-V). *Q) Hembra adulta, postabdomen, la flecha indica el pecten medio. R) Hembra adulta, base de la garra posabdominal con tres peines dorsales (proximal y medio indicados por flechas) y un grupo de dientes ventrales robustos. S) Hembra adulta, quinto toracópodo. T) Hembra adulta, segundo toracópodo. U) Hembra adulta, exópodo del segundo toracópodo. V) Hembra adulta, gnatobase del segundo toracópodo. Las barras de escala indican 0.1 mm (A-G, J), 10 µm (K-M, Q, R), 5 µm (H, N, P) y 25 µm (S-V).*

<u>Thoracopods (Th)</u>

Th1 (Fig. 4A) – distal lobe or endite 4 with single anterior thicker setulated seta, and two soft setae. Endite 3 with short, strong, single anterior seta (arrow), bearing noticeable lateral spinules-like projections in both sides, and single long and strong posterior seta. Endite 2 with two posterior setae. Endite 1 with three posterior setae. Two ejector hooks of remarkably different sizes. No maxillary process on limb base.

Th2 (Figs. 4B-D) – with large cylindrical lobe bearing long apical seta, its distal part regularly setulated. Distal endite with two setulated setae, other two endites with only one setulated seta each. One small seta near gnathobase plate (Fig. 3T, Fig. 4B, D, I), followed anteriorly by small hook-like outgrow (arrow in Fig. 3U, Fig. 4D: se). Rest of gnathobase margin with row of setae arranged fan-like (Fig. 3V). A long beating seta (see Kotov *et al.*, 2005) and a neighboring more anteriorly located, long seta (1 in Fig. 4B) separated by large gap (Fig. 3T). Both unilaterally setulated with long peg-like setules facing proximally. After these two long setae are a row of 10 to 12 gradually shortening and curved filter setae, with a fan-like arrangement (a-l in Fig. 4B). This group of setae is closed by a group of three anterior setae, inserted in the basal corner of the gnathobase (Fig. 4C: 2-4). , curved proximally, one with unilateral setulation facing distally (Fig. 4C: 4), and two more reduced brush-like (Fig. 4C: 2-3).

Th3 – exopodite large, flat, with four distal setae (c-f in Fig. 4E) and two short proximal setae, one short and one long (a and b in Fig. 4E), all of them bilaterally setulated. Inner distal portion with three endites. Endite 3 with single anterior seta (3 in Fig. 4E); endite 2 with one

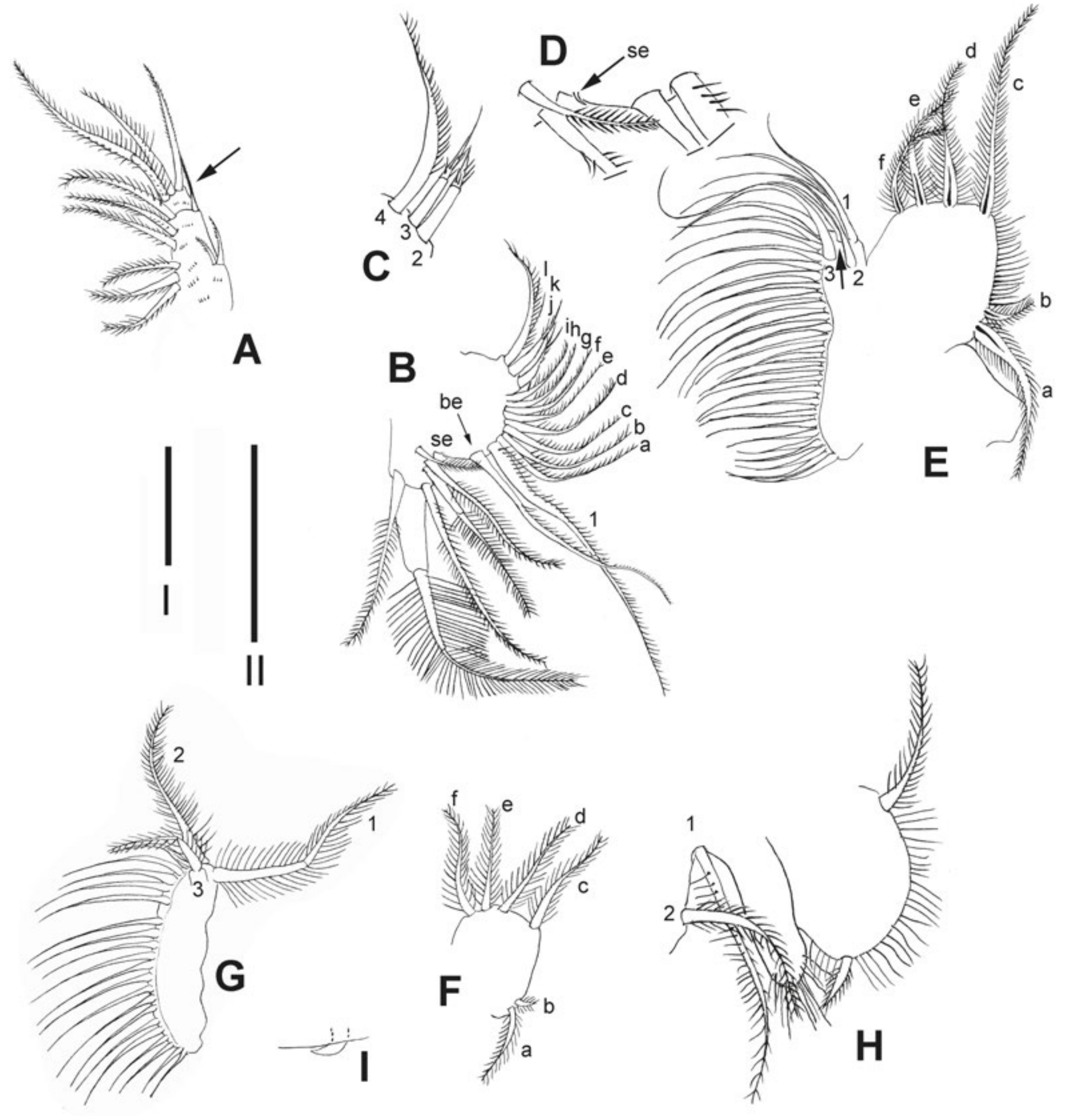

Figure 4. Thoracic limbs of female of *Moina micrura* s. str. (drawings based on individuals from Sádka pond at Nové Dvory). A: Th1 general view; B: Th2 general view; C: Th2 – anterior setae; D: Th2 – detail of the small seta and sensilla (se); E: Th3 general view; F: Th4, exopod; G: Th4, gnathobase; H: Th5, general view; I: Th2, detail of the sensilla. Scale bars indicate 50 µm (bar I: A, B, E-G; bar II: H-I). *Apéndices torácicos de la hembra de* Moina micrura *s. str. (dibujos basados en individuos del estanque Sádka en Nové Dvory). A: Th 1 vista general; B: Th2 vista general; C: Th2 - setas anteriores; D: Th2 - detalle de la seta pequeña y sensila (se); E: Th3 vista general; F: Th4, exópodo; G: Th4, gnatobase; H: Th5, vista general; I: Th2, detalle de la sensila. Las barras de escala indican 50 µm (barra I: A, B, E-G, barra II: H-I).*

anterior and one posterior seta (2, and arrow in Fig. 4E). Endite 1, with one anterior seta (1, in Fig. 4E). Rest of endopodite with 28-29 soft setae (Fig. 4E).

Th4 (Figs. 5G) – exopodite like Th3, but more elongated (Fig. 4F), with four long setae (c-f in Fig. 4F) in distal part and two setae in proximal part, one short and other long (a-b in Fig. 4F). Inner distal portion of thoracopod with two endites: endite 1 with long articulated and bisetulated seta (1 in Fig. 4G). Endite 2 with two long setulated setae (2, 3 in Fig. 4G). Filter plate with 20-22 setae (Fig. 4G).

Th5 – with large ovoid lobe, with setulated margin, large distal seta and small proximal seta, both bi-setulated (Fig. 3S and 4H). Inner thoracopod portion with elongated lobe with long setulae followed by two setae (1-2 in Fig. 4H).

Postabdomen – elongated, conically narrowing distally. Ventral margin almost straight, with numerous rows of minute setules in transversal rows (Fig. 3Q). Large anus located closer to base of postabdomen than to its distal extremity, preanal margin long, straight. Post-anal part tapering to claws. Preanal angle not well expressed. Laterally, row of 4-5 large, triangular teeth, fringed with marginal setules. Rows of setules on distal portion continue along base of bident tooth; distal branch of bident tooth significantly longer than proximal branch.

Setae natatoriae longer than postabdomen; basal part naked, distal part with two rows of fine setules (Fig. 1).

Postabdominal claw slightly curved, with sharp, pointed tip (Fig. 3Q, R). Three successive pectens along dorsal margin: basal pecten consisting of 4 to 8 slender spines huddled together, followed by middle pecten of 7-12 strong, thick spines; distal pecten consisting of smaller spines forming row from mid-claw to naked tip (Fig. 3Q: arrow). Basal and middle pectens fused in some individuals. Uniform row of spinules along inner face of claw. 4-7 large denticles on ventral margin, near base of claw.

Ephippial female (Figs. 3B, D-G)

Ephippium broadly rounded, tapering only slightly in posterior part. Central part over egg chamber embossed. Dorsal ridge strongly sclerotized (Fig. 3D: arrow, 3E: right arrow). Lateral surface with marked hexagonal reticulated pattern (Fig. 3F, G). Ultrastructure of ephippial surface (magnification up to 1000x – 2000x) formed by complex structure of polygons interlocked with many thin tentacle-like projections of various length. Some projections cover also inner surface of polygons (Fig. 3G).

Male (Fig. 3C, 3H-M)

Body – smaller than female, body length 0.45-0.54 mm (n = 10), more elongated as compared to female (Fig. 3C). Dorsal margin of valves almost straight, postero-dorsal angle clearly distinct. Antero-ventral portion of valves fringed with long, thin setules. Denticles along posterior margin more distinct than in female, arranged in groups (Fig. 3J: arrow, 3K).

Head – more elongated than in female, rostrum absent, labrum less fleshy than that of female. Optical vesicle fills top of head. No trace of pigment in ocellus.

Antennule – long, antennular sensory seta relatively long, male seta and sensillum inserted in first third of antennule (Figs. 3H). Nine aesthetascs and three to four hooks of distinct size and orientation, with bifurcated tips, present distally (Figs. 3I).

Antenna – similar in armature to female. Distal margin of all segments with row of thick, short spinules.

Carapace surface without any hairs or setules (Fig. 3J).

Th1 – with thick copulatory hook, its tip with two spinules, endite 4 (IDL) with three setae, endite 3 with one seta. Basal part short and rounded (width: length ratio about 1:1), with row of stout bristles on ventral face (Figs. 3L). Small wart-like outgrow at inner base of hook (Fig. 3L, arrow).

Postabdomen – as in female, shorter, with about 4-5 setulated teeth, distalmost tooth thin and with sharp tip (Fig. 3M). Distal bident tooth as in female. About 4-5 teeth in basal pecten of postabdominal claw, up to 10 in middle pecten (arranged fan-like) and 5-7 on ventral side at basal part of claw. Gonopores in lateral position

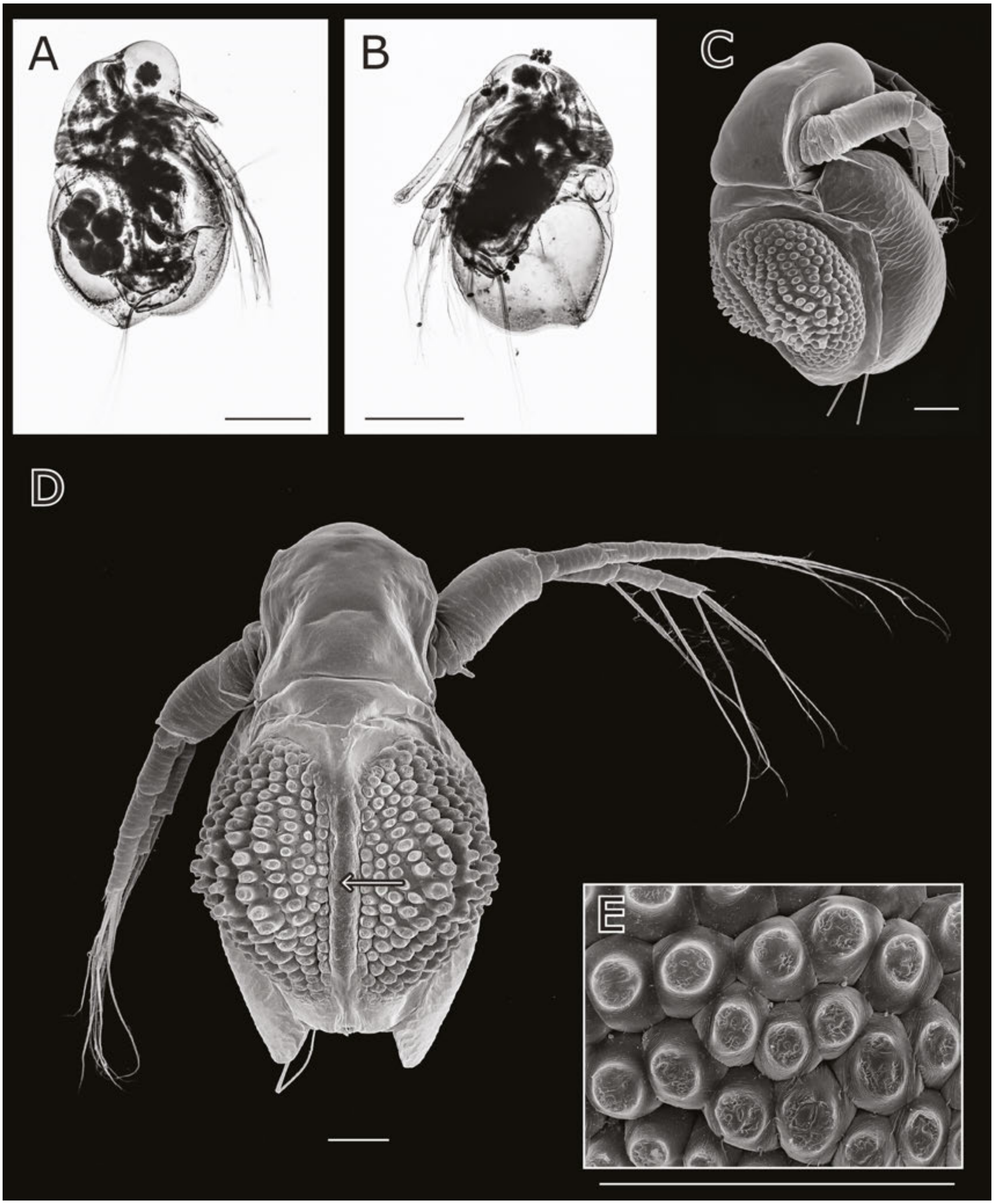

Figure 5. Morphological features of *Moina weismanni*. Specimens sampled at Radíkovice, Czech Republic (03.07.1957, images A & B) and at Krasnodar, Russia (21.07.1952, all other images). A-B: light microscope, focus stacked images. A) Adult female in lateral view. B) Adult male in lateral view. C-M: SEM. C) Adult ephippial female in lateral view. D) Ephippial female in dorsal view. Dorsal ridge marked with arrow. E) Ephippium ultrastructure. *Características morfológicas de* Moina weismanni*. Muestras recolectadas en Radíkovice, República Checa (03.07.19573, julio de 1957, imágenes A y B) y en Krasnodar, Rusia (21.07.195221 de julio de 1952, todas las demás imágenes). A-B: microscopio óptico, imágenes apiladas. A) Hembra adulta en la vista lateral. B) Macho adulto en la vista lateral. C-M: SEM. C) Hembra efipial adulta en vista lateral. D) Hembra efipial en vista dorsal. La flecha señala la cresta dorsal. E) Ultraestructura del efipio.*

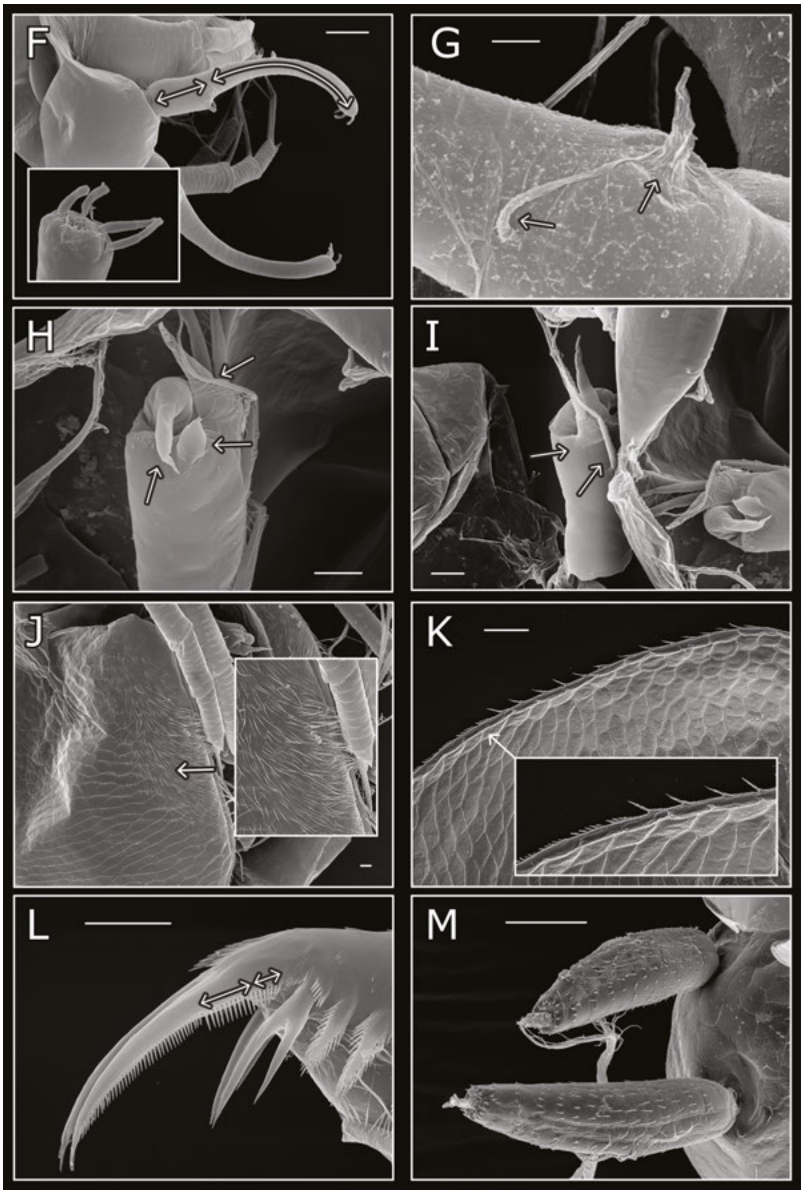

Figure 5 (cont.). F) Adult male, antennule (detail of tips in inset). Arrows show the proportion of the antennular proximal and distal parts. G) Adult male, sensory setae inserted in the first quarter of the antennule length; see arrows. H) Adult male, first thoracopod, ventral aspect. Left arrow marks small copulatory hook, middle arrow marks broad scale-like seta, and top right arrow the thick and naked seta in the background. I) Adult male, first thoracopod in dorsal aspect. Upper arrow indicates thick seta; lower arrow points the lateral seta, J) Adult male, carapace with short hairs. Detail shown in inset. K) Adult female, carapace ventral margin. Detail shown in inset. L) Adult female, postabdominal claw. Arrows indicate –proximal and middle pecten of dorsal slender teeth. M) Adult female, first antennae. Scale bars indicate 0.1 mm (A-E) and 10 µm (F-M). *F) Macho adulto, primera antena (detalle de las puntas en el recuadro). Las flechas muestran la proporción de las partes proximal y distal de la anténula. G) Macho adulto, setas sensoriales, insertadas en el primer cuarto de la anténula (ver flechas). H) Macho adulto, primer toracópodo, aspecto ventral. La flecha izquierda marca un pequeño gancho copulatorio, la flecha del medio - marca una seta con aspecto de escama, y la flecha de arriba a la derecha señala la seta gruesa y desnuda en el fondo. I) Macho adulto, primer toracópodo, aspecto dorsal. La flecha superior indica la seta gruesa, la inferior señala la seta lateral, J) Macho adulto, caparazón con pelos cortos. El detalle se muestra en el recuadro. K) Hembra adulta, margen ventral del caparazón. El detalle se muestra en el recuadro. L) Hembra adulta, garra postabdominal. Las flechas indican los pecten dorsales proximal y medio con dientes finos. M) Hembra adulta, primera antena. Las barras de escala indican 0.1 mm (A-E) y 10 µm (F-M).*

Table 2. Differential diagnosis between *Moina micrura* Kurz and *Moina weismanni* Ishikawa. *Diagnosis diferencial entre* Moina micrura *Kurz y* Moina weismanni *Ishikawa.*

Character	***Moina micrura***	**Fig.**	***Moina weismanni***	**Fig.**
Female				
Shape of antenna I.	straight	4P	curved	6M
Spines in middle pecten on postabdominal claw	short, robust; 1:4 *)	4R	long, slender; 1:11-12 *)	6L
Ephippial surface	reticulated	4F,G	distinctly raised knobs	6C,D,E
Ephippial ultrastructure (SEM)	polygons interlocked with many tentacle-like projections	4G	distinct knobs without projections	6E
Male				
Anterior surface of carapace	Naked	4J	covered with dense "fur" of short hairs	6J
Length of basal part of the 1st antenna	about 1/3 of its length	4H	About ¼-1/5 of its length	6F
Setae along anterior ventral margin	about same length		growing in length distally	
1st thoracopod	short basal part (without hook); 1:1 *)	4,L	long basal part; 1:2 *)	6H,I
	hook robust	4L	hook feebly developed	6H
	small outgrow at base of hook	4L	broad scale-like seta at base of hook	6H

* Ratio widthlength (width of spines in pecten measured at their base)

situated close to proximal triangular tooth (Fig. 3M, arrow).

Differential diagnosis

Within *terra typica*, the species *Moina micrura* may be confused probably with only one other species: *Moina weismanni* Ishikawa, 1896 (Figs. 5A-D). Both species frequently co-occur in the same habitats and their parthenogenetic females look superficially similar. The list of easily recognized individual characters which differentiate *M. weismanni* from *M. micrura* is provided in Table 2. Full differential diagnosis from other members of the *micrura*-like species complex requires detailed systematic revision of populations from other biogeographic regions and the lineages detected by molecular methods.

Intercontinental and interspecific comparison of *Moina* ephippial ultrastructure

Specific ultrastructure on the ephippial surface from the *terra typica* (Fig. 3G), irregular hexagonal pillow-like cells, observed on the material from the Sádka pond, is morphologically similar to the structure observed on the material from other European localities (see Supplementary information, Table S2). However, we did not observe such structure on any of the *M. micrura* s. l. material from Africa, Asia, South America or Australia (Fig. 6A-H), whose ephippial structures vary from very flat hexagonal porous patterns (e.g., Figs. 7C, H) to well-delineated polygonal shapes (Fig. 6G). Ephippial ultrastructure of other *Moina* species (Fig. 7A-H) was even more variable, ranging from almost smooth surfaces (Figs. 7A, B) to rounded cells (Figs. 7G, H) or even prominent protuberances (Fig. 5D-E).

DISCUSSION

Moina micrura has been one of the most poorly defined cladoceran species, recorded under that name in almost any part of the world from the temperate regions to the tropics. Even worse, animals identified as *M. micrura* have been widely used as models for culturing or as ecotoxicology biomarkers. Results of studies employing this taxon, however, cannot be directly compared or replicated, as mentioned in the introduction. There was thus an urgent need to establish the correct assignment for *M. micrura* as a valid species, including the neotype, genetic analyses and detailed morphological description of all forms within the life cycle.

According to the International Code of Zoological Nomenclature (International Commission on Zoological Nomenclature 1999), designation of a neotype is justified when there is an exceptional need to clarify the taxonomy of a species (as is the case when several poorly defined sibling species exist), or when there is a reasonable certainty that the original type material does not exist. In case of *M. micrura*, both these conditions are met. Our search for the type material of *M. micrura* was unsuccessful. Kurz's original samples or specimens are not available either in the National Museum, Prague (curator's personal communication, P. Dolejš, 2014) or in Naturhistorisches Museum, Wien (curator P. Dworschak, 2015), which would be most likely institutions for depositing types of animal species described in the 19th century from Bohemia.

Considering the documented existence of several genetically well-defined lineages, so far labelled as *Moina micrura*, it is necessary to establish the neotype and the new type locality as a reference for future studies. We chose the new type locality as close as possible from the original one studied by Kurz (1875). However, typical *M. micrura* localities in *terra typica*, the watersheds of the Elbe and Danube rivers, are fish ponds and other relatively small water bodies with rapidly changing environments. Therefore, it is highly probable that many present-day *Moina* populations will disappear or re-occur in the near future. Hence, we added some more localities from *terra typica* as a comparative material.

Despite rapidly changing natural environments of Europe, we managed to collect *M. micrura* no more than 10 km from the Kurz's original type locality. Petrusek *et al.* (2004) used material located about 90 km from the *locus typicus*; to confirm the conspecifity of our population we sequenced the gene for 12S rRNA originally studied by Petrusek *et al.* (2004). Both studied Czech populations indeed belonged to the

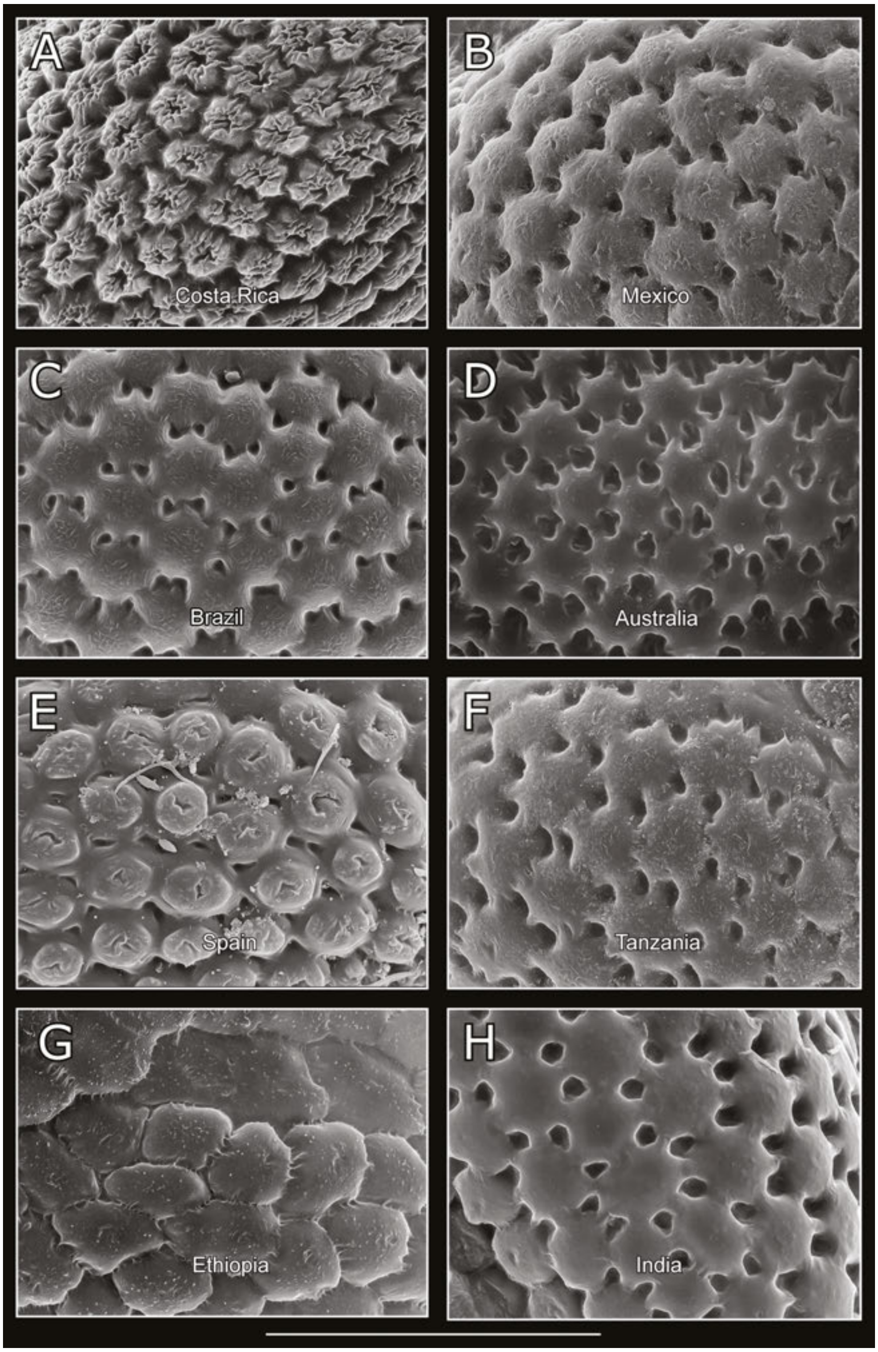

Figure 6. Intercontinental comparison of ephippial ultrastructure of *Moina micrura* s. l. Ephippia were mostly positioned in the ephippial female. All images are of the same magnification. A) Costa Rica (18.05.1981). B) Bordo La Colorada, Aguascalientes, Mexico (18.01.1989). C) Lima, Lima Campos, Ceara, Brazil (23.05.1989). D) Relfs, NSW, Australia (25.01.1968). E) Lake Albufera, Valencia, Spain (2015). F) Mwanza, Tanzania (07.04.1985). G) Borena, Oromia, Ethiopia (01.10.1983). H) Jabaddi Ludhiana, Punjab, India (13.09.1977). Scale bar indicates 0.1 mm for all photographs. *Comparación intercontinental de la ultraestructura efipial de* Moina micrura *s. l. Los efipios se tomaron principalmente en la hembra. Todas las imágenes son con la misma magnificación. A) Costa Rica (18.05.1981). B) Bordo La Colorada, Aguascalientes, México (18.01.1989). C) Lima, Lima Campos, Ceara, Brasil (23.05.1989). D) Relfs, NSW, Australia (25.01.1968). E) Albufera de Valencia, España (2015). F) Mwanza, Tanzania (07.04.1985). G) Borena, Oromia, Etiopía (01.10.1983). H) Jabaddi Ludhiana, Punjab, India (13.09.1977). La barra de escala indica 0.1 mm para todas las fotografías.*

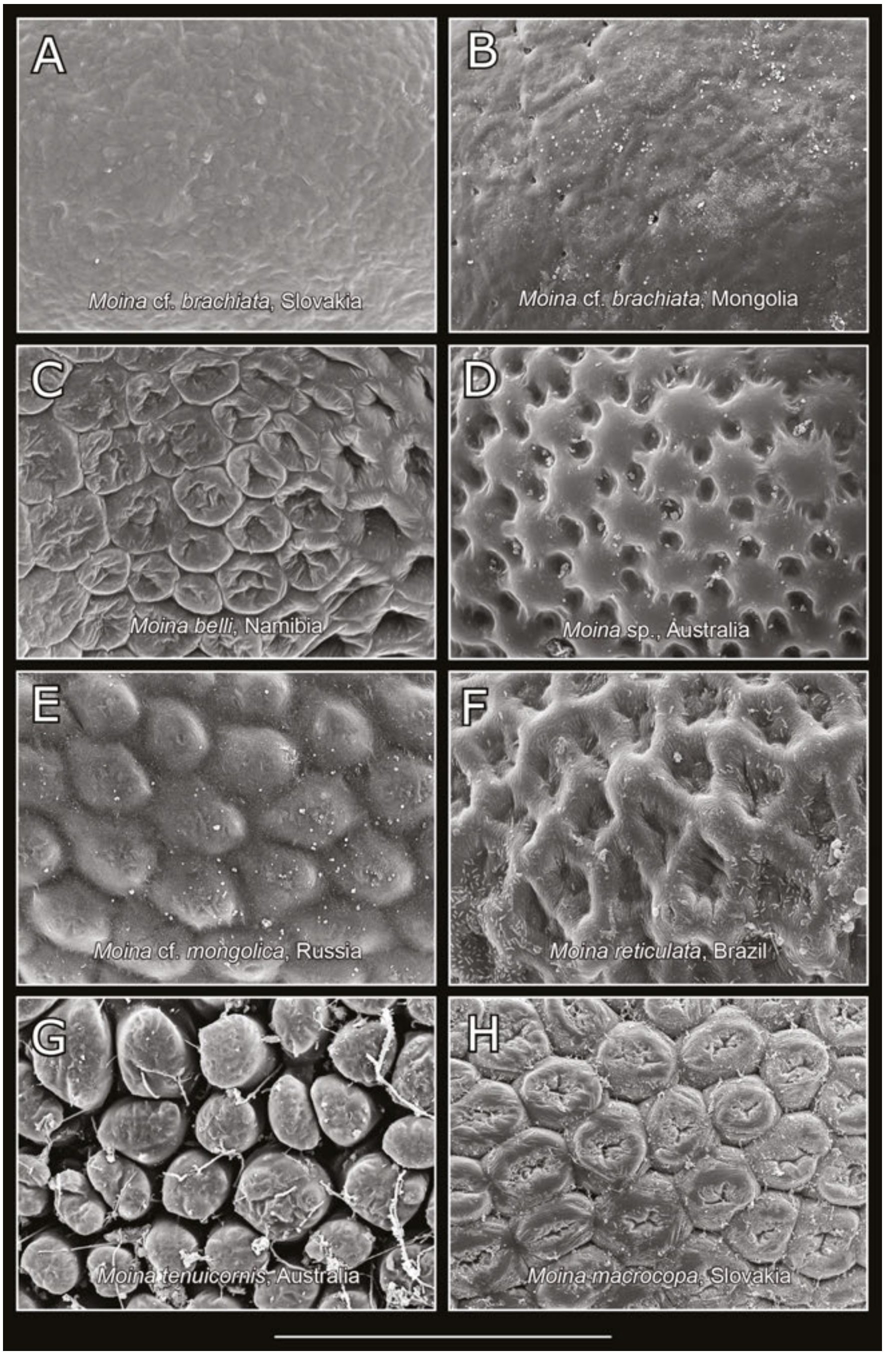

Figure 7. Variation of ephippial ultrastructure among different *Moina* species. All images are of the same magnification. A) Bač, Slovakia (27.07.1954). B) Yihe Modoto, Mongolia (06.09.1988). C) Avis Dam, Namibia (27.04.1972). D) North Victoria highway, Australia (05.02.1982). E) Schantrapay, Russia (26.07.1974). F) Lago Jacateringa, Brazil (02.09.1980). G) Alexandria, Australia (26.09.1973). H) Komárno, Slovakia (27.07.1954). Scale bar indicates 0.1 mm for all photographs. *Variación de la ultraestructura efipial entre diferentes especies de* Moina*. Todas las imágenes son con la misma magnificación. A) Bač, Eslovaquia (27.07.1954). B) Yihe Modoto, Mongolia (06.09.1988). C) Presa de Avis, Namibia (27.04.1972). D) Carretera al norte de Victoria, Australia (05.02.1982). E) Schantrapay, Rusia (26.07.1974). F) Lago Jacateringa, Brasil (02.09.1980). G) Alexandria, Australia (26.09.1973). H) Komárno, Eslovaquia (27.07.1954). La barra de escala indica 0.1 mm para todas las fotografías.*

same species, and thus provide a solid base for future comparisons.

The geographical distribution of *M. micrura* s. str. remains unclear, but the available data (based on both genetic analyses and morphological comparisons) suggest that this species is restricted to Europe and the western part of the Palearctic. Apart from Central Europe, genetic data confirmed the presence of the same lineage also in the Middle East (Israel) and the Ponto Caspian region (Kazakhstan). Molecular analyses nevertheless showed that within Eurasia, numerous other lineages, presumably species, of the *M. micrura* complex reside (Bekker *et al.*, 2016; this study), this is also true for Europe alone where genetically distinct lineages were detected in Hungary or Spain. Sequenced American (Elías-Gutiérrez *et al.*, 2008; Prosser *et al.*, 2013) and Australian populations (Petrusek *et al.*, 2004) compared with all Eurasian sequences indicate that they are all distinct species as well, with K2P divergences at COI or 12S exceeding 13 % and 8 %, respectively. The distinctness of divergent clades of the complex are also supported by a hybridization experiment that demonstrated an apparent reproductive isolation between a European and an Australian clone (Petrusek *et al.*, 2004).

A detailed redescription of morphology of *M. micrura* s. str. presented here provides a necessary step for disentangling of this rich species complex. Furthermore, we highlight a potential importance of ephippial ultrastructure for *Moina* systematics. This feature that was for long suspected as taxonomically relevant: Goulden (1966) suggested the possibility that the structure of the ephippial surface in *Moina* is crucial in the male's selection of the partner during mating, and is thus species-specific. As available identification keys cover presently-known members of the family Moinidae only partially, we have selected 15 gamogenetic populations of *Moina micrura* s. l. from available samples collected on different continents, in which ephippia were present. To assess further the diversity of ephippial ultrastructures within the genus *Moina*, we added SEM pictures of nine additional species distinct from *micrura*-like populations (Figs. 6 and 7). Comparison of the ephippial surfaces revealed several distinct patterns of ornamentation. None of them resembles that of *Moina micrura* Kurz, 1875 and the feature seems to be a good differential character in species identification. Such substantial variation in the ephippial ultrastructure seems to support the Goulden's original hypothesis, although the potential role of this morphological character feature in establishing or maintenance of reproductive barriers between *Moina* species needs testing. In any case, these apparently species-specific characteristics of *Moina* ephippia open a possibility to develop keys for their routine identification in various limnological projects.

Most of above-mentioned morphological traits have not been described before, at least for the *micrura* complex. Future analysis of them is recommendable, to understand the evolution, adaptation and success of these organisms in different regions of the world. Any detailed study of these complexes also requires a detailed knowledge of the males and ephippial female morphology, because these often present more relevant species-specific traits to recognize the species than parthenogenetic females (as also shown for, e.g., *Daphnia*; Juračka *et al.*, 2010; Popova *et al.*, 2016). Although gamogenetic populations may occur only sporadically in the field, there is a possibility to induce sexual individuals in a laboratory culture, including hormonal stimulation to obtain males (Kim *et al.*, 2006).

Combining detailed studies of morphology, with distribution data and molecular markers have been very useful to detangle complex groups of sibling species (Popova *et al.*, 2016). The *M. micrura* complex would deserve a similar treatment in the future, redescription of *M. micrura* s. str. thus should be the beginning only.

Sequences from the COI gene will likely play a useful role in future identifications, as molecular techniques are becoming widespread even in routine biodiversity surveys and limnological projects. Unfortunately, in most cases when possible hidden taxa of cladocerans are uncovered by molecular tools, no nomenclatural act is realized, often due to the lack of reference material to compare (e.g., Elías-Gutiérrez *et al.*, 2008), or due to the focus of the study other than taxonomy (e.g., Xu *et al.*, 2016). However, to foster further research and appropriately recognize the diversity of groups in question in other biological fields (e.g., ecotoxicology), formally naming well-defined lineages, even cryptic species, should be considered (Fišer *et al.*, 2018).

CONCLUSIONS

We can conclude that *Moina micrura* s. str. is distributed at least from Central Europe (with *terra typica* in the Czech Republic) to the Ponto Caspian region and Middle East but it seems to overlap in distribution with other related species (as a genetically distinct lineage has been detected in Hungary). *Moina* cf. *micrura* from Albufera Lake in Spain and Sobrón reservoir possibly is another, not yet described species that requires further attention. Is clear that European *Moina micrura* s. str. is distinct from analyzed populations from North and South America, Australia, Africa, Korea or Russia. The *M. micrura*-like populations from American continent are a complex of species, consisting of at least five distinct lineages that also require further, more detailed analyses. We hope this work, based on integrative taxonomy, establishes a baseline for future comparisons of *M. micrura* s. str. with other members of this complex, in order to facilitate its critical revision and the delimitation of species within.

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Moina macrocopa (Straus, 1820): a species complex of a common Cladocera, highlighted by morphology and DNA barcodes

Lucía Montoliu-Elena[1,2], Manuel Elías-Gutiérrez[2,*] and Marcelo Silva-Briano[3]

[1] Posgrado en Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México; Av. Ciudad Universitaria 3000, Coyoacán 04510, Ciudad de México, México.
[2] El Colegio de la Frontera Sur, Chetumal unit, Av. Centenario Km 5.5, Chetumal 07714, Quintana Roo, México.
[3] Laboratorio No.1, Ecología. Edificio 202. Centro de Ciencias Básicas. Av. Universidad No. 940 Ciudad Universitaria. Universidad Autónoma de Aguascalientes, Aguascalientes 20131, México.

[*] Corresponding author: melias@ecosur.mx

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ABSTRACT

***Moina macrocopa* (Straus, 1820): a species complex of a common Cladocera, highlighted by morphology and DNA barcodes**

Freshwater microcrustaceans are one of the most diverse and understudied groups of the animal kingdom and are best described by the use of an integrative taxonomic approach, including morphological, molecular and geographical data. In this study, we used three different types of characters, including detailed morphological analyses, DNA barcodes (COI gene) and geographical distribution to study the Old-World clade *Moina macrocopa* (Straus, 1820), here considered as *M. macrocopa* s.l. and the American clade *Moina macrocopa americana* Goulden, 1968. Results have indicated that these clades are different species and not subspecies, while *Moina macrocopa* s.l. is suggested to be species complex with a minimum of three different clades.

Key words: zooplankton, *Moina macrocopa*, cryptic species, integrative taxonomy, DNA Barcodes, COI

RESUMEN

Moina macrocopa *(Straus, 1820): complejo de especies en un cladócero común, destacado por su morfología y códigos de barras de ADN*

Los microcrustáceos de aguas continentales son uno de los grupos más diverso y menos estudiado del reino animal. Un enfoque integrativo, que incluya al menos, datos morfológicos, moleculares y geográficos es esencial para delimitar las especies de estos invertebrados, como es el caso del complejo Moina macrocopa*. En este estudio, utilizamos tres tipos de caracteres: morfológicos, genéticos (códigos de barras del ADN) y geográficos, para demostrar que* Moina macrocopa *(Straus, 1820), el clado del Viejo Mundo, considerado aquí como* M. macrocopa *s.l., y* Moina macrocopa americana *Goulden, 1968, el clado americano, son especies distintas, no subespecies. Además, confirmamos que* Moina macrocopa *s.l. es un complejo de especies, formado por un mínimo de 3 clados diferentes.*

Palabras clave: *zooplancton,* Moina macrocopa*, especies crípticas, taxonomía integrativa, Códigos de Barras de la Vida, COI*

INTRODUCTION

Moina is one of the most confusing genera of cladocerans and is the second most diverse Anomopod (Goulden, 1968; Elías-Gutiérrez *et al.*, 2008a; Nédli *et al.*, 2014; Bekker *et al.*, 2016).

This cladoceran has been mainly recorded in water bodies severely eutrophicated under heavy anthropogenic influences (sewage, fertilizers). It is a species whose populations play a key role in food webs of epicontinental aquatic environments (Vignatti *et al.*, 2013) and it is of economic importance due to its use as live food for fishes (Valdivia-Villar, 1988; Elías-Gutiérrez, 1995) and as bioindicator of water quality (Nandini *et al.*, 2004).

Moina macrocopa macrocopa (Straus, 1820) was drawn for the first time by Joblot in 1754 who referred to it as "pou aquatique" (water flea) (Joblot, 1754). 65 years later, in 1819, the French zoologist Hercule Eugène Straus-Durckheim illustrated this species again, and finally, in 1820, he described it as *Daphnia macrocopus* (Straus, 1820). After his publication, numerous synonyms have been described due to the lack of details in his description and illustration (Straus, 1819, 1820; Goulden, 1968) and it was not recognized as its currently valid name *Moina macrocopa* until the early 1900's (Goulden, 1968).

Goulden (1968) in his revision of the genus, proposed two subspecies: *Moina macrocopa macrocopa* (Straus, 1820) as the typical form restricted to the Old World, and described *Moina macrocopa americana*, type locality Cheyenne Bottoms Waterfowl Refuge in Barton County, Kansas, limited to USA. Several morphological differences have been found between both subspecies, being the first clues of the non-cosmopolitanism of Cladocera suggested later by Frey (1980, 1987) and afterward presented by Bekker *et al.* (2016; Fig. 2).

Arevalo (1920), cited *Moina macrocopa macrocopa* (Straus, 1820) for the first time in the Iberian Peninsula, from Gandía (Comunidad Valenciana, Spain) under the name *Moina rectrirostris var. casañi*. Then, Alonso (1996) on his revision of the Iberian Fauna, described and illustrated *Moina macrocopa macrocopa* (Straus, 1820) from Albufera de Valencia (Comunidad Valenciana, Spain), being the only place in the Iberian Peninsula where he found this species. This subspecies was then reported in many localities all over the world in: Europe, Asia, South America and North Africa (Goulden, 1968; Elías-Gutiérrez & Zamuriano-Claros, 1994; Paggi, 1997; Petrusek, 2002; Mangas-Ramírez *et al.*, 2004; Elmoor-Loureiro *et al.*, 2010; Kotov *et al.*, 2012; Rietzler *et al.*, 2014; Ghaouaci *et al.*, 2018).

In the case of the American subspecies, *Moina macrocopa americana* Goulden, 1968, thought its distribution restricted to the United States but in 2011, it was recorded from Canada (Manitoba) (Jeffery *et al.*, 2011), and previously it was found in central Mexico (Elías-Gutiérrez, 1995; Elías-Gutiérrez *et al.*, 2008b; Prosser *et al.*, 2013) expanding its distribution area all over North America.

Due to the ubiquity, small size, phenotypic plasticity, genetic variability, cryptic taxa and lack of taxonomical identification keys for many parts of the world (Goulden, 1968), an integrative approach must be made to delimitate with accuracy the species from this complex. The use of DNA barcoding (cytochrome oxidase c subunit 1 – COI gene as a fast, reliable and cost-effective tool to delimit the animal species (Hebert *et al.*, 2003a) has led to the discovery of cryptic diversity among invertebrates (Mills *et al.*, 2016; Chertoprud *et al.*, 2017 and Lavinia *et al.*, 2017) and other animal groups (i.e. Álvarez-Castañeda *et al.*, 2012; Lima *et al.*, 2017). In this regard, Bekker *et al.*, (2016) have demonstrated with DNA barcodes, a possible high cryptic diversity of the Old World *Moina macrocopa* (Straus, 1820), though they only presented a tree with differences among Eurasian clades without discussing it. For this reason, we hereby designate all specimens from the Old World as *M. macrocopa* s.l. (sensu lato).

Other authors proved the value of DNA Barcoding for the early detection of exotic species in many groups (Valdez-Moreno *et al.*, 2012; Gutiérrez-Aguirre *et al.*, 2014; Montoliu *et al.*, 2015). Finally, the biogeographical signal of this gene shows a high correspondence between haplotypes and restricted distributions permitting us to delimitate with accuracy the species (Mills *et al.*, 2016).

In this work, we studied the *Moina macrocopa* species complex using morphological, genetical (DNA Barcodes) and biogeographical analyses to re-describe *Moina macrocopa americana* Goulden, 1968 and demonstrate that it is a distinct species, not a subspecies, of *Moina macrocopa* s.l.

MATERIAL AND METHODS

Sampling

Specimens of *Moina macrocopa* s.l. and *Moina macrocopa americana* Goulden, 1968 were collected with a plankton net with mesh size of 45 µm, and a hand net with mesh size of 90 µm, from Albufera Lake in Valencia (Spain), a small pond in Calderitas (Quintana Roo, Mexico), Texcoco Lake (Mexico State, Mexico), Los Gringos dam and Niagara dam (Aguascalientes, Mexico) (see table S1, available at http://www.limnetica.net/en/limnetica). All samples were preserved in 96 % alcohol (non-denaturated) following the procedure suggested by Prosser *et al.* (2013) and stored in the freezer for a minimum duration of 72 h, to preserve the DNA.

Morphological observations

Specimens were sorted from the ethanol samples under a stereomicroscope and placed in a drop of glycerol. Ten females and males of each population were dissected. Whole animals and dissected sections were examined and measured under a compound microscope, and identified following the descriptions of several authors, i.e., Straus (1820), Goulden (1968), Alonso (1996), Paggi (1997), Elías-Gutiérrez *et al.* (2008b). Five specimens of each sex and subspecies were prepared for Scanning Electron Microscopy (SEM) for the observation of microcharacters with a JEOL microscope Model JSM6010 Plus in Chetumal Unit of ECOSUR (Mexico) at 10 KV and a JEOL microscope Model LB5900 at 12 KV in the Autonomous University of Aguascalientes (Mexico).

Mexican specimens were deposited in the reference collection of El Colegio de la Frontera Sur (ECOSUR) Chetumal unit, in Mexico, and Spain, in the collection of Freshwater zooplankton at the Institut Cavanilles de Biodiversitat i Biologia Evolutiva (University of Valencia, Spain).

Molecular markers

COI gene was selected as a molecular marker for this study for its fast-evolving mitochondrial protein-coding genes, which reflect the evolutionary history of invertebrate populations (Audzijonyte & Väinölä, 2006). Also, it is a useful tool to discriminate cladoceran species, becoming a standard for species-level identification (Machida *et al.*, 2004; Dasmahapatra *et al.*, 2010; Gutiérrez-Aguirre *et al.*, 2014; Montoliu *et al.*, 2015), and a model in species delimitation (Puillandre *et al.*, 2012).

DNA isolation, PCR amplification, and sequencing

DNA was extracted using AcroPrep 96 Filter Plate 3 µm GF/ 0.2 µm BioInert (Pall Corporation, Port Washington, NY, USA) according to the manufacturer's instructions. The Polymerase Chain Reaction (PCR) was used to amplify approximately 600-658 bp of the COI gene using Zplank primers suggested by Prosser *et al.* (2013). The 12.5 µl PCR reaction mixes included 6.25 µl of 10 % trehalose stabilizer, 2 µl of ultrapure water, 1.25 µl of 10X PCR buffer, 0.625 of MgCl2 (50 mM), 0.125 µl of each primer (0.01 mM), 0.0625 µl of each dNTP mix (0.05 mM), 0.625 µl of Taq polymerase (5 U/µl) (New England Biolabs or Invitrogen), and 2.0 µl of DNA template. Thermocycler program was as follows: 94 °C for 1 minute, 5 cycles of 94 °C for 40 seconds, 45 °C for 40 seconds, 72 °C for 1 minute, followed by 35 cycles of denaturation at 94 °C for 40 seconds, annealing at 51 °C for 40 seconds, elongation at 72 °C for 1 minute, and a final extension at 72 °C for 5 minutes, with a final hold at 4 °C. The PCR products were visualized on a 2 % agarose gels (E-gel® 96 system, Invitrogen Inc.). Amplicons were bidirectionally sequenced using BigDye Terminator Cycle Sequencing Kit (v3.1) on an ABI 3730XL DNA Analyzer. The forward and the reverse sequences were assembled, edited and aligned using Codon-

Code Aligner v. 5.0.1 (CodonCode Corporation, USA) and tested in MEGA6 (V 6.0) (Tamura *et al.*, 2013) to verify that they were free of stop codons and gaps.

We prepared a dataset under the name DS-MMACRO with all specimens and sequence information in the Barcode of Life (BOLD). It includes all sequences generated in this study and all public sequences available and previously published, including those in GenBank.

Sequence analysis

COI sequences obtained in this study were combined with those for *M. macrocopa* s.l. and *M. macrocopa americana* Goulden, 1968 available in GenBank and BOLD (see table S1, available at http://www.limnetica.net/en/limnetica) to corroborate the identity of our sequences and to gain a better understanding of the geographical genetic diversity for both subspecies.

COI sequences were aligned with BOLD aligner and the non-overlapping sequence regions at the 5' - and 3' -ends were trimmed.

As the first approach, genetic divergence between groups was calculated using K2P model (Kimura, 1980) with pairwise deletion of gaps and missing data.

To perform the Maximum Likelihood (ML) tree distances, a best-fitting model of nucleotide substitution was selected in MEGA6, based on the likelihood for 24 different nucleotide substitution models and the Akaike information criterion (Posada & Buckley, 2004). The best model was Tamura 3-parameters with a specific fraction of sites evolutionarily invariable (T92+I) (Tamura, 1992). The analysis was performed using 500 bootstraps, and partial deletion of gaps and missing data. Finally, a single sequence of *Moina* cf. *micrura 2* from Mexico was used as an outgroup, (See table S1, available at http://www.limnetica.net/en/limnetica). Monophyly was confirmed with the web service "Monophilizer" (Mutanen *et al.*, 2016).

Species delimitation analysis

Molecular operational taxonomic units (MOTU's) have frequently been used to infer putative species boundaries where morphological identifications are difficult (Ashfaq *et al.*, 2015). To assess the presence of cryptic taxa, we implemented four different clustering algorithms to assign the COI sequences from *M. macrocopa* s.l. and *M. macrocopa americana* complex species to MOTU's: the Refine Single Linkage algorithm (RESL, Barcode Index Number, or BIN) (Ratnasingham & Hebert, 2013), Automatic Barcode Gap Discovery (ABGD) (Puillandre *et al.*, 2012), multi-rate Poisson tree processes (mPTP) (Triantafyllidis *et al.*, 2011) and statistical parsimony networks (TCS) (Clement *et al.*, 2002).

The BIN system uses the RESL algorithm to reach decisions on the number of MOTU's in a sequence dataset through a three-phase analysis based on sequence variation with a 2.2 % threshold of maximum divergence allowed within a cluster.

ABGD employs a multi-phase system which initially divides sequences into MOTUs based on a statistically inferred barcode gap (i.e., initial partitioning), and subsequently conducts additional rounds of splitting (i.e., recursive partitioning). It is a statistical method that explores the distribution of all pairwise distances looking for the gap between intra- and interspecific distances. An online version of ABGD (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.htm) was employed using default parameters and K2P as the distance metric.

The mPTP is a multi-rate Poisson tree processes for single-locus species delimitation under maximum likelihood and Markov chain Monte Carlo (Kapli *et al.*, 2017). For this analysis, also an online version was used (http://mptp.h-its.org/#/tree).

Finally, TCS applies the statistical parsimony method to construct haplotype networks. It has been shown that the count of Linnaean species present in a COI alignment greatly matches that of independent statistical parsimony networks inferred by the software. A nexus file was generated to calculate a TCS network (Clement *et al.*, 2002) in PopART (http://popart.otago.ac.nz) (Leigh & Bryant, 2015). This Interface permits to visualize in a graph the network of relationships between the haplotypes and geographical distributions.

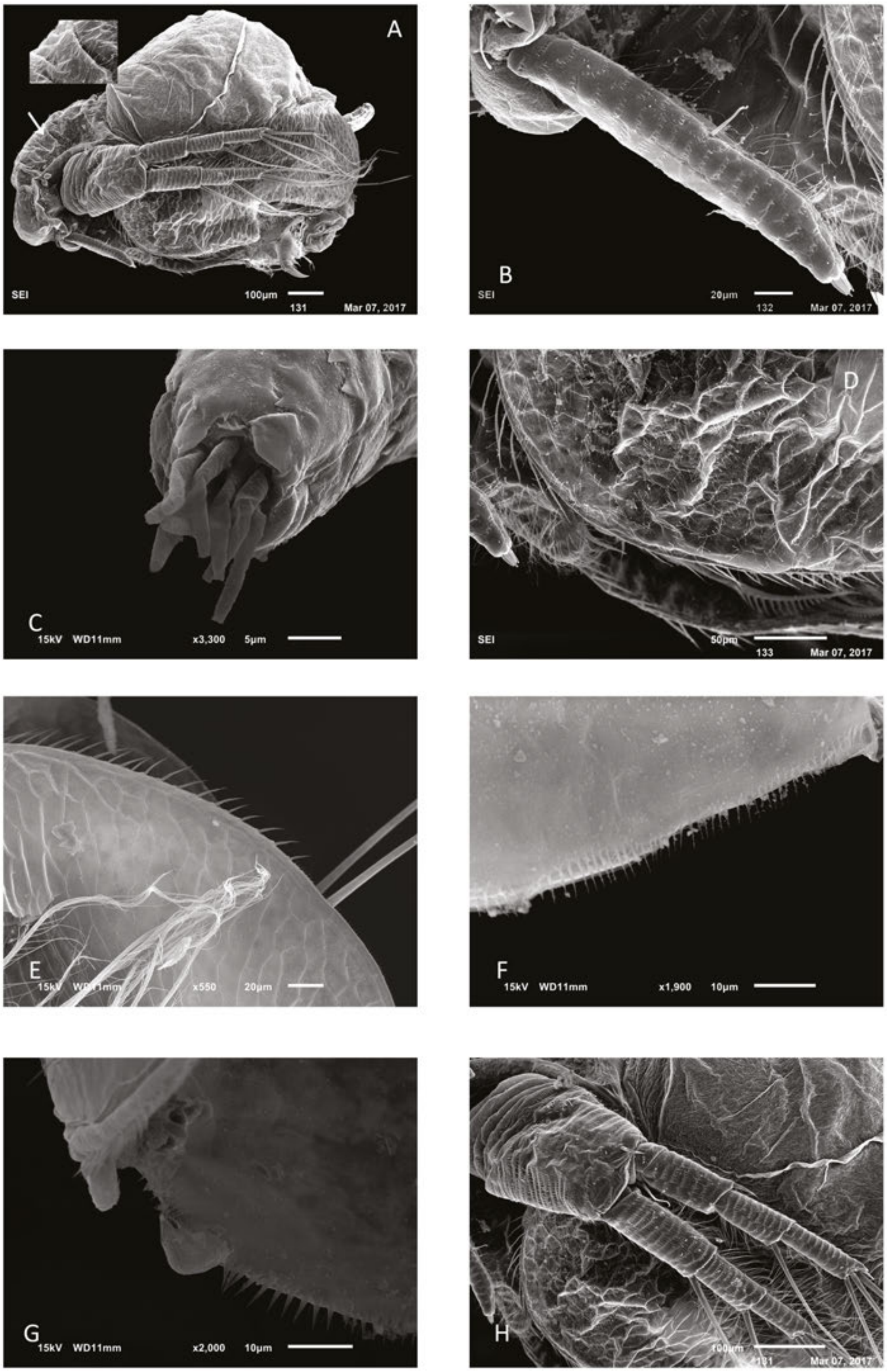

Figure 1. SEM observations of *Moina macrocopa* s.l. (Female). A. Habitus, arrow: magnification of hairs. B. Antennule. C. Tip of the antennule. D. Anterior shell rim and first limb. E. Posterior shell rim, exterior view. F. Posterodorsal shell rim, interior view. G. Dorsal shell rim and hooks. H. Antenna. *Observaciones de* Moina macrocopa *s.l (hembra) con el Microscopio Electrónico de Barrido. A. Hábito. B. Anténula. C. Punta de la anténula. D. Margen anterior del caparazón y pata I. E. Margen posterior del caparazón, vista exterior. F. Margen postero-dorsal del caparazón, vista interior. G. Margen dorsal del caparazón y ganchos. H. Antena.*

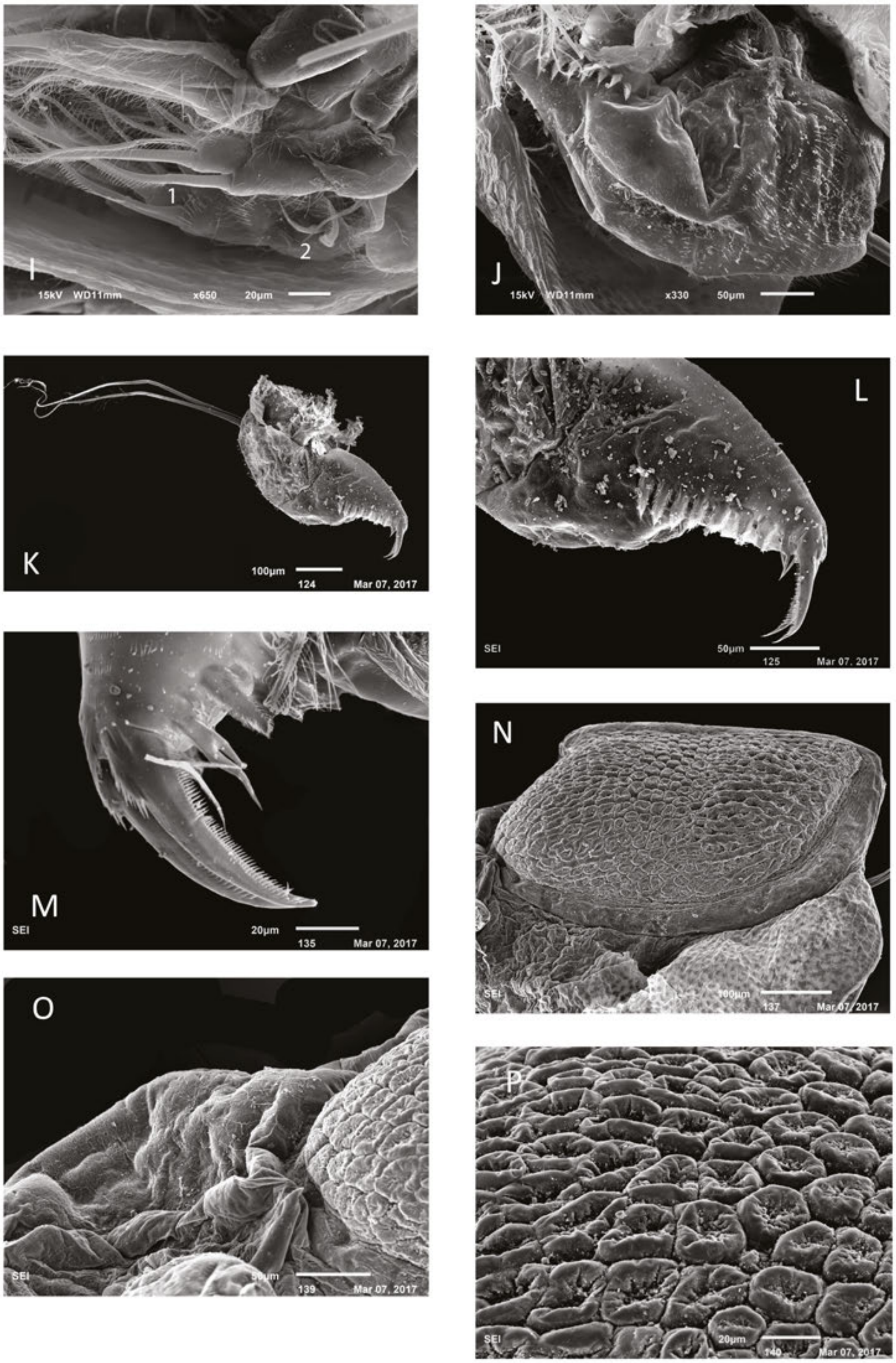

Figure 1 (cont.). *Moina macrocopa* s.l. I. First limb, 1: toothed seta, 2: ejector hooks. J. Postabdomen. K. Postabdomen and swimming setae. L. Claw and feathered teeth. M. Claw and pecten. N. Ephippium, lateral view. O. Head and ephippium. P. Surface of ephippium. M. macrocopa *s.l. I. Pata I, 1: seta dentada, 2: ganchos eyectores. J. Postabdomen. K. Postabdomen y setas natatorias. L. Garra y dientes plumosos. M. Garra y pecten. N. Efipio, vista lateral. O. Cabeza y efipio. P. Superficie del efipio.*

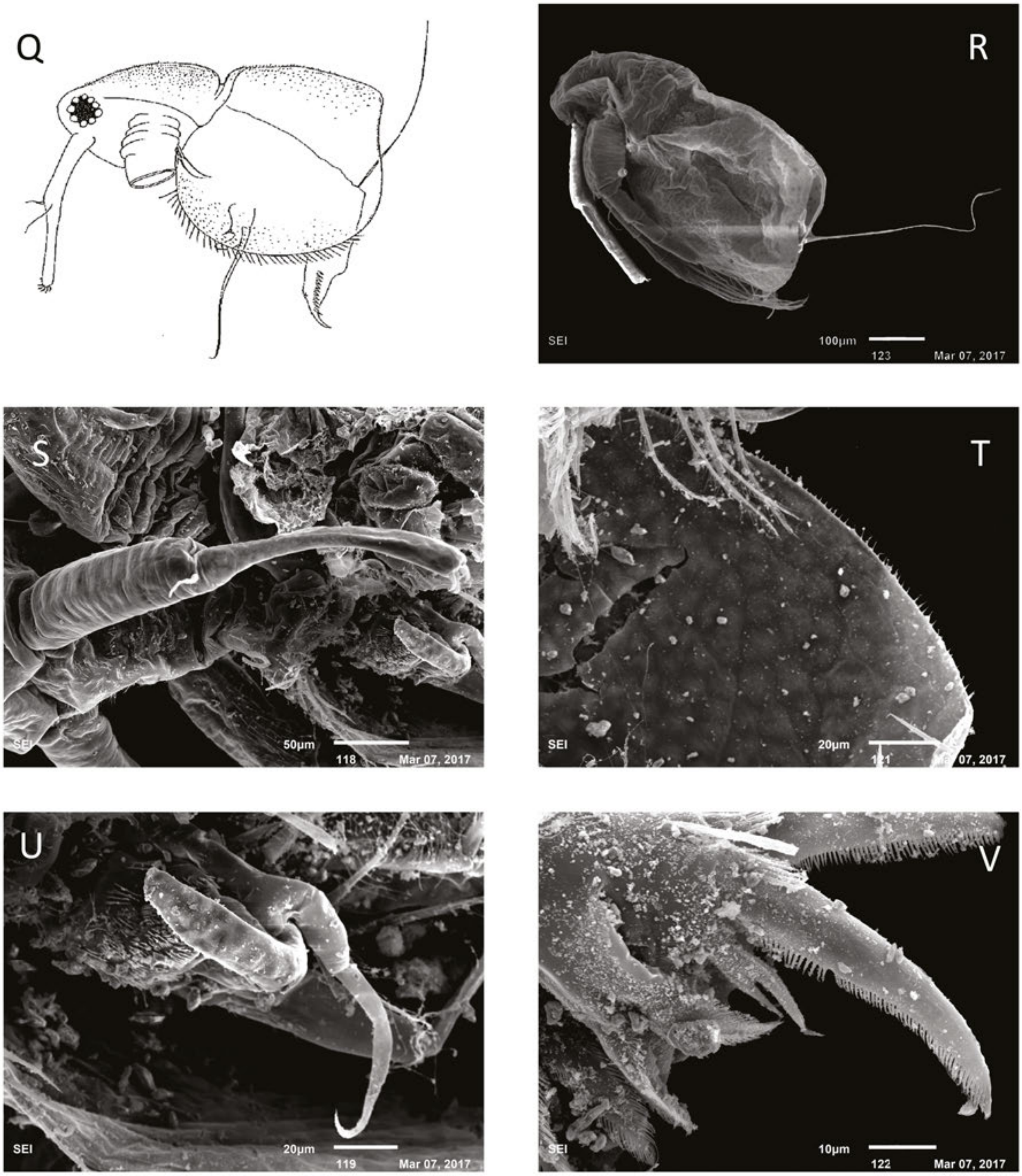

Figure 1 (cont.). *Moina macrocopa* s.l. (Male). Q. Habit, illustration of Alonso (1996). R. Habit. S. Antennula. T. Posterior shell rim. U. First limb, inmature form. V. Claw. All specimens for SEM are from Calderitas (Mexico). Moina macrocopa *s.l. (Macho). Q. Hábito, ilustración de Alonso (1996). R. Hábito. S. Anténula. T. Margen posterior del caparazón, vista interior. U. Pata I, forma inmadura. V. Garra. Todos los especímenes para Microscopía Electrónica de Barrido (MEB) fueron recolectados en Calderitas (México).*

Genetic diversity analysis

The alignments in FASTA format were imported into DnaSP v5 to perform haplotype diversity indexes and verify results obtained by TCS network (Librado & Rozas, 2009).

RESULTS

Morphological observations

Family Moinidae Goulden, 1968

Genus *Moina* Baird, 1850

Moina macrocopa (Fig. 1A-P)

Synonymy uncertain

Type locality: unknown, Europe. Considering that the majority of the species described by Straus where from France, as *Daphnia magna* (Straus, 1820) and the proximity of France (*terra typica*) and Spain, we consider that the population shared between Spain, Russia and Calderitas (Mexico) corresponds to the real *Moina macrocopa* s. str. (Straus, 1820).

Material examined: One parthenogenetic female from Albufera de Valencia (Spain) (32.296 N, 0.316 W). 10 specimens from a rain pond in Calderitas (Quintana Roo state, Yucatan Peninsula, Mexico) (18.6308 N; 88.2251 W) probably recently introduced.

Parthenogenetic female

Size: Mean: body length 1.3±0.103 mm; body wide 81±0.08 mm; body ratio (length/wide): 0.656±0.008 mm (N = 10). Color of specimens: brownish.

The morphological features of the Mexican specimens from Calderitas and those from Spain match with the typical form (Fig. 1). The head and shell surface are covered with scattered long hairs, the head broad with a slightly supraocular depression (Fig. 1A, arrow) and there is an oval "nucal pore" close to the dorsal end of the head, similar to *Moina americana* Goulden, 1968 n. stat. (Fig. 2B,C). These characters were also examined in the Argentinean and Spanish specimens of Paggi (1997) and Alonso (1996), respectively, but were not mentioned by Goulden (1968), although it is possibly the most significant character of the species complex, since it has not been reported in other congeners as i.e. *Moina micrura*.

The shell is deeply reticulated (visible at SEM) with hairs appearing on the border of the polygons (Fig. 1D). The hairs are denser at the anterior part of the shell and disappear at the dorsal part. The number of setae in the ventral rim of the shell ranges from fifty-five to seventy (Fig. 1D,E), the ¬posterior edge has a submarginal, continuous row of fine spinules (Fig. 1E,F), and at the dorsum there is a pair of hooks, one on each valve (Fig. 1G). The setae of the anterior rim are slightly feathered. The antennule is cylindrical (mean length: 0.214 mm; mean ratio width/length: 0.22), ciliated, slightly curved on the tip and in its surface has many short rows of spinules around it (Fig. 1B). At the tip of the antennule, there are nine aesthetascs (only eight visible at Fig. 1C) with flat tip and without projections as in *Moina micrura*. The sensory seta is long, but not as long as described in Goulden (1968) and Paggi (1997), and it is inserted in the mid-point of the antennule (Fig. 1B). The antennae are robust, covered with many rows of spinules. Both branches have medial rows of long and fine setulae and the usual distribution of swimming setae (1-1-3/0-0-1-3) and spines (0-0-1/0-1-0-1). The sensory setae of the basipodite are approximately equal in length, about one-third the length of the segment. The basipodite covered by a row of spines parallel to it, and one stout spine at the tip (Fig. 1H).

The first trunk limb has the setation pattern common to most species of *Moina* (3-2-2-3), eight feathered setae, two toothed setae, and two ejector hooks (Fig. 1I). The anterior seta of the penultimate segment has a row of stout and distal teeth that may be longer than the seta's width. (Fig. 1I). The teeth on the seta of the last segment are much smaller but higher in number and closer than the penultimate tooth seta. The limb II presents the two setae of the proximal group of the gnathobase of the same size as described in Alonso (1996). We did not consider it as a significant taxonomical feature because it is similar in other congeners and does not present any variation in the studied populations. In Limb IV Alonso (1996) cited that the setae of the filter plate are relatively separated than the other congeners, but we did not find any significant difference with other congeners i.e. populations of *Moina micrura* complex (pers. obs.).

The postabdomen is long and robust with many short rows of spinules at the dorsal and lateral sides (Fig. 1J) with two biarticulated natatory setae with long hairs at the distal part (Fig. 1K). The conical distal part of the postabdomen carries 7-10 feathered teeth, as Goulden (1968) described instead of 9-11 teeth cited by Paggi (1997), and one bifid tooth (Fig. 1L). The branches of the bifid tooth are distinctly unequal. There are some scattered tiny spinules near the base of the bifid tooth. The inner side of the claw has a continuous row of fine setae a little thicker than those on the outer side. On the ventral base, there is a pecten with seven teeth (Fig. 1M) only visible at SEM.

The surface of the ephippium is covered with thick rounded cells and contains two eggs (Fig. 1N-P). It is sub rectangular in lateral view (Fig. 1N).

Male (Fig. 1Q-U)

Size: main body length 0.52 mm (N = 5). Color of specimens: brownish.

The body male and head are covered with hairs (Fig.1Q,R), longer and denser on the body. The head lacks the supraocular depression. The long antennules originate below the eye, are bent at the mid-point and have two sensory setae arising at or near the knee of this bend (Fig. 1S). The distal halves of the antennules are curved inward and have four to six short hooks at the tip. The hooks form a semicircle around a group of sensory papillae that project from the end of the antennule. The surface of the shell is reticulated and covered with hairs, as mentioned above. The ventral rim has 35-40 marginal setae. The shorter spines along the posterior margin of the shell are ungrouped and scattered as in the female (Fig. 1T).

The first limb of the male has a long, recurved hook originating from the penultimate segment (Fig. 1U). The terminal segment carries three setae; the middle seta is long and hook-like. The other two setae are feathered. The penultimate segment covered with many short hairs along the medial margin, and there is a seta arising from this surface opposite the hook. The first limb has

Table 1. Morphological differences between species and sexes. *Diferencias morfológicas entre especies y sexos.*

Characters	***Moina macrocopa* (Straus, 1820)**	***Moina americana* Goulden, 1968 n. stat.**
Female		
Antennulae (A1)	Sensory seta inserted in the middle	Sensory seta longer, inserted in the first third
Setae of posterior shell rim	No groupings	Forming three groups
Setae of dorsal shell rim	Fine spinules	Stout and robust spinules
Toothed seta of LI	Fine and separate teeth	A row of stout teeth, smaller and more numerous
Ephippium	Round cells	Trapezoidal cells
Male		
Limb I	Large recurved hook	Very large recurved hook
Genital openings	Ventral to the claw	At the end of the feathered teeth

an exopod segment that terminates with a very long seta which is longer than the limb and reaches to the posterior margin of the shell. This seta is usually bent ventrally and projects well beyond the ventral margin as in the review of Goulden (1968). The postabdomen and the claw of the male are similar to the female. However, the conical part of the postabdomen is much broader, and the claw projects from the middle of the distal margin of the postabdomen (Fig. 1V).

Habitat: In Spain, this species has only appeared in the Albufera of Valencia, and related water bodies (Arévalo, 1920; Alonso, 1996) such as the rice fields at the south part of the lake. In these rice fields, the quality and stationarity of the water mass depend on the rice cultivation cycle. During the summer waters are transparent, but loaded with organic matter from rice fertilizers, shallow (about 25 cm depth) and come from Jucar River, so they are always renewing. In Calderitas (Mexico), we collected a possibly recently introduced specimens, from a temporary pond formed by the rain, with a high organic load.

Moina americana Goulden, 1968 n. stat. (= *Moina macrocopa americana* (Goulden, 1968))

Type locality: According to Goulden (1968), a roadside ditch, along a county road one mile east of the Cheyenne Bottoms Waterfowl Refuge in Barton County, Kansas (USA). Sequenced specimens from near terra typica were collected in a pond in Denver (40.73 N, -104.40 W).

Type specimens of this subspecies are in the Smithsonian Institution National Museum of Natural History (Catalogue Numbers Holotype 123203; Paratypes 123204).

Material examined: 15 specimens from Texcoco Lake (State of Mexico, Mexico) (19.45 N, -99.00 W) and 10 specimens from Los Gringos dam (Aguascalientes, Mexico) (21.91 N, -102.26 W).

Diagnosis (see Table 1)

Moina americana n. stat. can be easily distinguished from *Moina macrocopa* s.l. for the following characters: the sensory seta of the antennule is longer, and it is inserted in the first third (Fig. 2D); the spines on the posterior rim of the valves form groups increasing in size (Fig. 2I). Following these spines at the dorsal part, there is a row of stout and robust spinules instead of fine spinules as is present in *Moina macocopa*; the first trunk limb presents the anterior seta of the penultimate segment with a row of stout teeth, finer and more numerous than in *M. macrocopa* s.l. (Fig. 2J). The ephippium is bigger than *M. macrocopa*, its surface covered with trapezoidal cells instead of round with the point slightly curved upwards (Fig. 2M-N). The male's first limb has a very large recurved hook, originating from the penultimate segment more prominent than in the Old World form and presents several tiny spinules on the tip (Fig. 3F).

Parthenogenetic female (Fig. 2A-L)

Size: Length 1.14±0.05 mm; width 0.83±0.05 mm and ratio: 0.729±0.005 (N = 10). Color of specimens: whitish. In life sometimes red, due to the presence of hemoglobin.

The revised material agrees with the typical form described by Goulden (1968): the setae along the posterior shell rim grouped, some being much larger than the others and there are usually two or three groups with large teeth located just posterior to the ventral row of setae. Long and dense hairs cover the body and head, more than the European clade (Fig. 2A,B). The head presents a broad and slightly supraocular depression and an oval "nucal pore", cited by Alonso (1996) and Paggi (1997) in the Old World form but never cited before for the American form (Fig. 2B,C). Labrum hairy, covered with many scattered fine and long hairs, the proximal part very bulky (Fig. 2F). The shells are sub rectangular and rounded in lateral view (Fig. 2G), with a row of stiff feathered marginal setae (Fig. 2H). The spines of the posterior rims are grouped and ordered in increasing in size (Fig. 2I) followed by a continuous row of fine spinules. The antennule is cylindrical, shorter than in *M. macrocopa* s.l. (mean length: 0.25 mm; mean ratio width/length: 0.188 mm), ciliated, slightly curved on the tip and in its surface presents long rows of spinules around it (Fig. 2D,E). The sensory seta is also longer than the European specimens analyzed and is inserted in the first third of the antennule (Fig. 2D). The antennae are robust, covered with many rows of

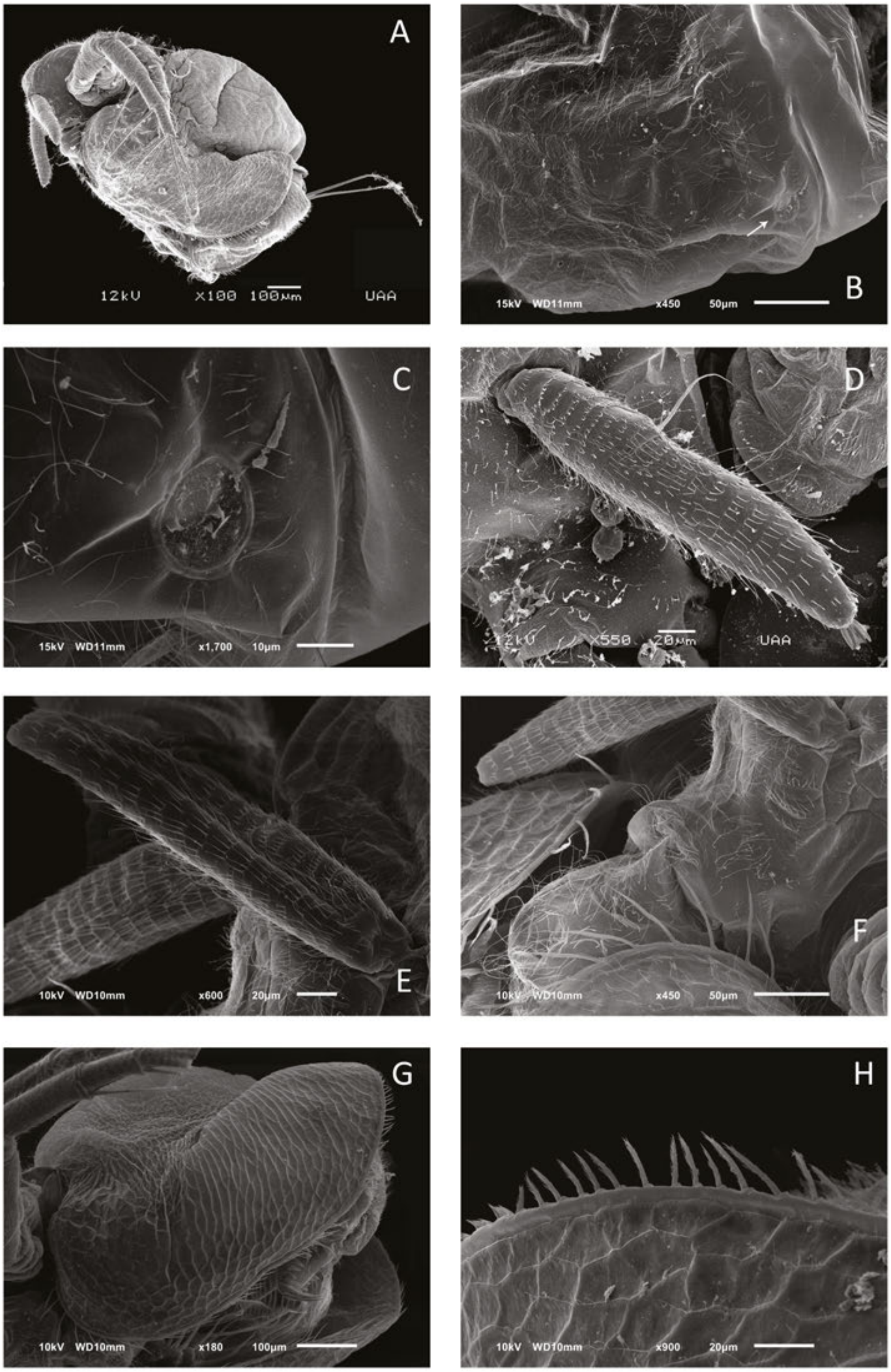

Figure 2. SEM observations of *Moina americana* Goulden, 1968 n. stat. (Female). A. Habit. B. Nucal pore on head. C. Magnification of the nucal pore. D, E. Antennula. F. Labrum. G. Shell, lateral view. H. Anterior shell rim. *Observaciones de* Moina americana *Goulden, 1968 n. stat. (Hembra) con el Microscopio Electrónico de Barrido. A. Hábito. B. Órgano nucal. C. Magnificación del órgano nucal. D,E. Anténula. F. Labro. G. Caparazón vista lateral. H. Margen anterior del caparazón.*

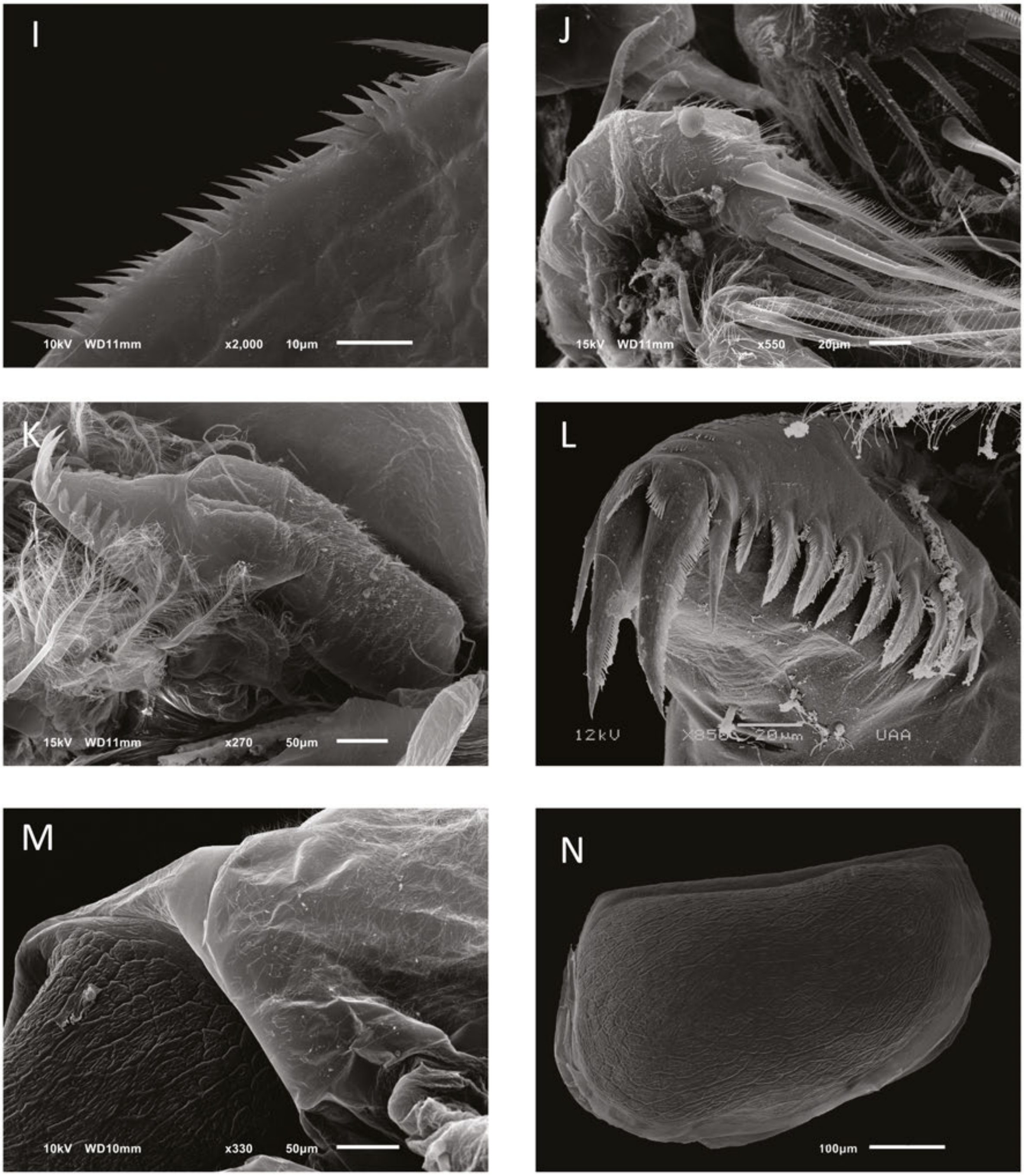

Figure 2 (cont.). *Moina americana* n. stat. I. Groups of setae present at the posterior shell rim. J. First limb. K. Postabdomen. L. Claw and feathered teeth. M. Head and ephippium. N. Ephippium, lateral view. Specimens for SEM are from Texcoco lake (City of Mexico) and Los Gringos dam (Aguascalientes, Mexico). Moina americana *n. stat. I. Grupos de setas presentes en el margen posterior del caparazón. J. Pata I. K. Postabdomen. L. Garra y dientes plumosos. M. Cabeza y efipio. N. Efipio, vista lateral. Especímenes para MEB recolectados en el Lago Texcoco (Ciudad de Mexico) y en la presa de Los Gringos (Aguascalientes, México).*

spinules. Both rami with medial rows of long and fine setulae and the usual distribution of swimming setae (1-1-3/0-0-1-3) and spines (0-0-1/0-1-0-1) (Fig.2A, H). The sensory setae of the basipodite are approximately equal in length, about half the length of the segment. The basipodite covered by a row of spines parallel to the segment, and one stout spine at the tip. (Fig. 2A).

The first trunk limb presents eight feathered setae, two toothed setae, and two ejector hooks of different size. The anterior seta of the penultimate segment has a row of stout teeth, smaller and more numerous than in *M. macrocopa* s.l. (Fig. 2J). The postabdomen is similar to the Old World specimens but hairier (Fig. 2K) It is very long and robust with many short rows of spinules at the dorsal and lateral sides. The conical distal part carries nine feathered teeth and one bifid tooth (Fig. 2L). The branches of the bifid tooth are distinctly unequal. There are small groups of spinules near the base of the bifid tooth. The inner side of the claw has a continuous row of fine setae a little thicker than those on the outer side. On the ventral base, there are thirteen teeth (Fig. L).

The surface of the ephippium composed of flat cells that are more trapezoidal then square in cross section and contains two eggs. It is bigger than in *Moina macrocopa* s.l. and seems like a water drop with the point slightly curved upwards (Fig. 2M,N).

Male (Fig. 3A-L)

The body male and head are hairy, lenght 0.92 mm (N = 10). The shell is deeply reticulated, the setules grow mainly in the contours of these polygons. The head lacks the supraocular depression (Fig. 3A,M) but also presents reticulation on dorsal view. The long antennules originate below the eye, they are bent at the mid-point and have two sensory setae arising at or near the knee of this bend (Fig. 3D). The distal halves of the antennules are curved inward with a row of tiny spinules on the tip and four to six short brush-like setae (Fig. 3E). The brush-like setae form a semicircle around a group of sensory papillae that project from the end of the antennule (Fig.3D). The antennae are robust, covered with many rows of spinules. Both rami with middle rows of long and fine setulae and the general distribution of swimming setae is 1-1-3/0-0-1-3 and spines 0-0-1/0-1-0-1. The sensory setae of the basipodite are approximately equal in length, about one-third the length of the segment. The basipodite covered by a row of spines parallel to the segment, and one stout spine at the tip. (Fig. 3G). The surface of the shell is reticulated and covered with hairs, as mentioned above (Fig.3A). The ventral rim has 34-43 marginal setae. The setae along the posterior margin of the shell are grouped and ordered in increasing size (see 1 in Fig. 3H) followed by a continuous row of fine spinules (Fig.3I). At the dorsal rim, there are some stiff spinules, stronger than in the European form, followed by two hooks (Fig. 3J).

The first limb of the male has a large recurved hook, originating from the penultimate segment, bigger than Old World specimens (Fig 3F). The terminal segment carries three setae; the middle seta is very long and hooklike without spinules on the tip (see a in Fig. 3F) as in other congeners as *Moina micrura*. The other two setae are feathered. The penultimate segment covered with many grouped short hairs along the medial margin, and there is a seta arising from this surface opposite the hook. The first limb has an exopod segment that terminates with a very long seta which is longer than the limb and reaches to the posterior margin of the shell and presents several little teeth on the tip (see b in Fig. 3F). These latter are absent in *M. macrocopa* s.l.

The postabdomen and the claw of the male, are similar to the female. However, the conical part of the postabdomen is much broader, and the claw projects from the middle of the distal margin of the postabdomen (Fig. 3K). The two genital openings are just at the end of the feathered teeth row, in lateral view, one on either side of the postabdomen not as in *Moina macrocopa* s.l. where they are ventral to the claws (see arrow and magnification in Fig. 3L).

Habitat: Los Gringos dam is in central highlands of Mexico, in the city of Aguascalientes (21.91 N; 102.268 W). The waters of this dam are highly eutrophicated due to the fact that it received the sewage water from the nearby neighborhood. Texcoco Lake is in the east of Mexico

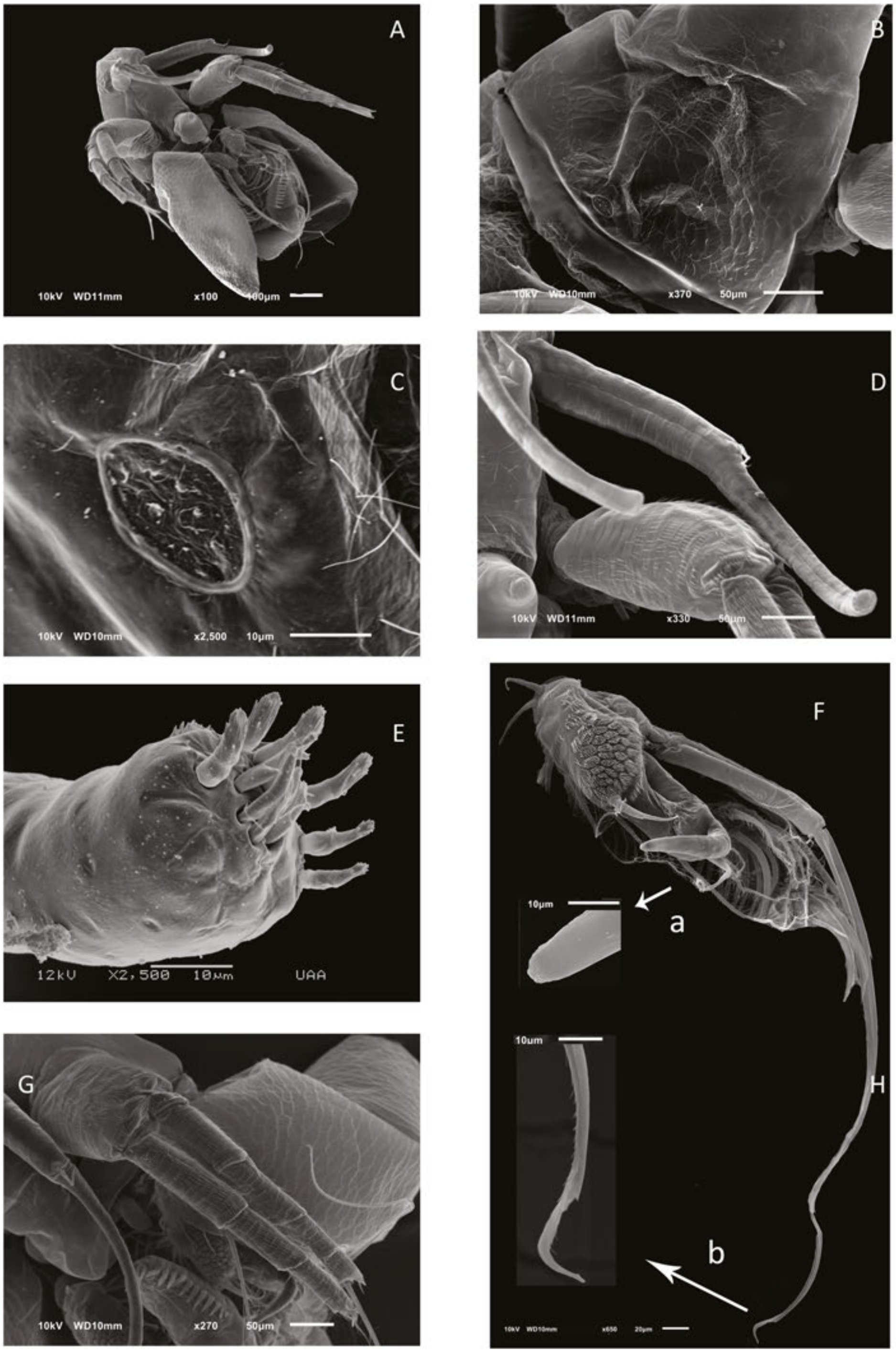

Figure 3. SEM observations of *Moina americana* Goulden, 1968 n. stat. (Male). A. Habit. B. Nucal pore on head. C. Magnification of the nucal pore. D. Antennules. E. Tip of the antenne, brush-like setae and aesthetascs. F. First limb, a: tip of the hook, b: tip of the middle seta of the penultimate segment. G. Antenne. *Observaciones de* Moina americana *Goulden, 1968 n. stat. (macho) con el Microscopio Electrónico de Barrido. A Hábito. B. Órgano nucal. C. Magnificación del órgano nucal. D. Anténulas. E, Punta de la antena, setas tipo brocha y estetascos. F. Pata I, a: detalle del gancho, b: punta de la seta del medio del penúltimo segmento. G. Antena.*

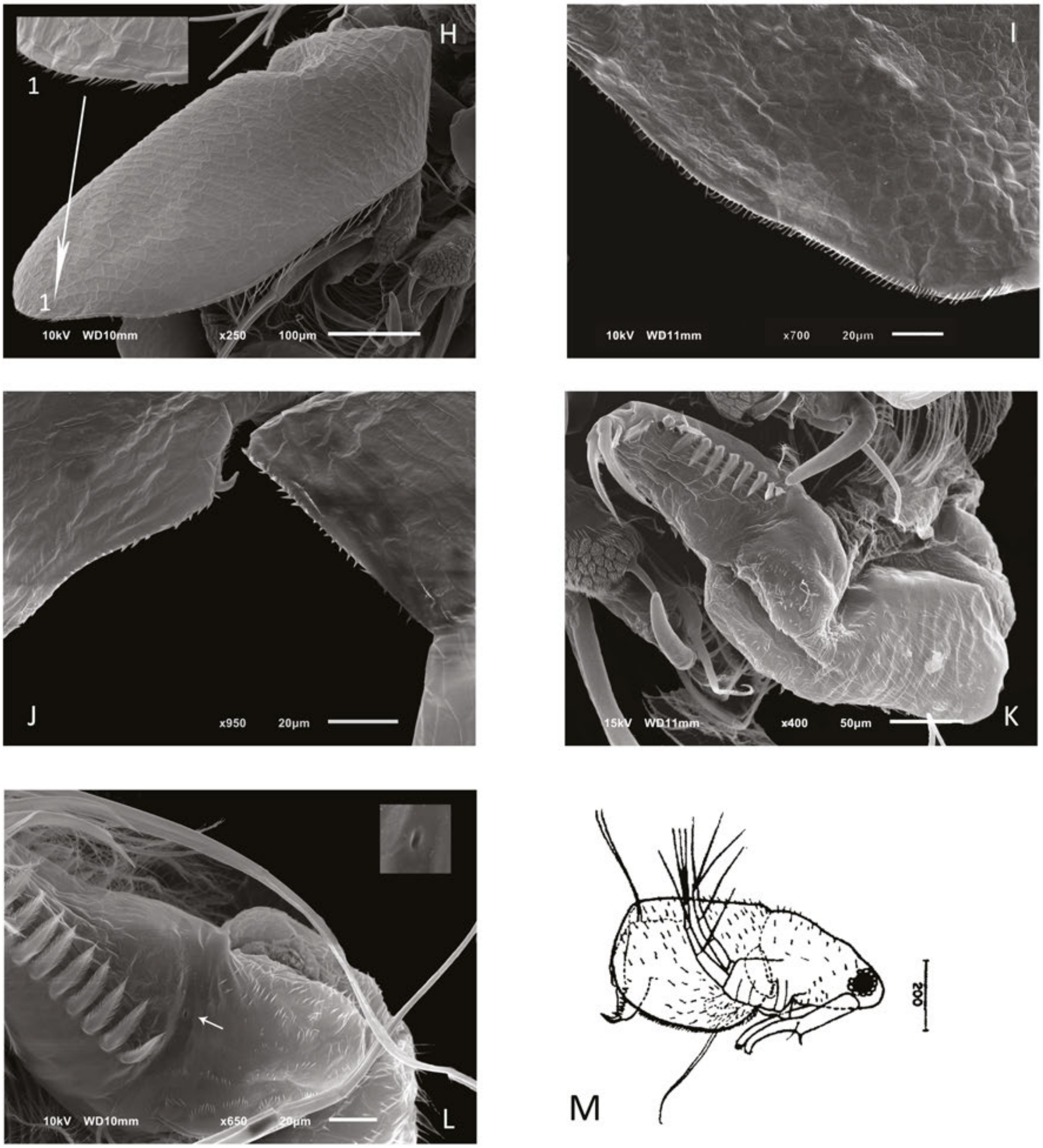

Figure 3 (cont.). *M. americana* n. stat. Male. H. Shell lateral view. I. Posterior shell rim, interior view. J. Dorsal shell rim and hooks. K. Postabdomen, claw and feathered teeth. L. Gonopores, arrow: shows the estructure. M. Drawing from Goulden (1968). Specimens for SEM from Texcoco Lake (City of Mexico). M. americana *n. stat. Macho. H. Caparazón, vista lateral. I. Margen posterior del caparazón, Vista interior. J. Margen dorsal del caparazón y ganchos. K. Postabdomen, garra y dientes plumosos. L. Gonoporos, flecha: muestra la estructura. M. Dibujo de Goulden (1968). Especímenes para MEB recolectados en el Lago Texcoco (Ciudad de Mexico).*

City (19.452 N; 98.995 W), located at 2.236 meters above the sea level in the TransMexican volcanic belt (Alcocer & Bernal-Brooks, 2010). This saline soda-lake is highly polluted and eutrophicated and supports an active anthropogenic pressure since the arrival of the Aztecs in the XIV century (Alcocer & Bernal-Brooks, 2010).

Remarks

We have found morphological differences between the two populations of the European clade inhabiting in Calderitas, such as the spinulation pattern of the posterior shell rim and the antennule, which have direct correspondence with genetical results commented later. But to describe this possible new species, further genetical and morphological comparative studies must be done.

Genetic analyses

We created a dataset of 55 sequences under the name of DS-MMACRO in BOLD Systems database. It includes 57 sequences of *Moina macrocopa complex* and one sequence of *Moina* cf. *micrura 2*, (Elías-Gutiérrez *et al.*, 2008a), used as an outgroup. The "barcode identification request" in BOLD showed five different BINs. Sequences matching European clade (possibly including the real *Moina macrocopa* (Straus, 1820), assigned to three different BINs, BIN: BOLD:ACH4664 (sampled from Hungary, Russia and Mexico, Calderitas), BIN: BOLD:ACA1705 (Spain, Russia and Mexico, Calderitas) and BIN: BOLD:AAK6825 (European Russia). Sequences matching the American clade, including specimens near the original type locality, *Moina americana* Goulden, 1968 n. stat. were assigned to a unique BIN: BOLD:AAC3108 and finally, sequences assigned to BIN: BOLD:ADF9261 correspond to the Asiatic clade found in Russia, shown by Bekker *et al.* (2016).

Marker	BIN System	mPTP Model	ABGD											
			0.0017		0.0028		0.0077		0.0129		0.0215		0.0359	
			I	R	I	R	I	R	I	R	I	R	I	R
COI	5	3	3	3	3	3	3	3	2	3	2	3	2	3

Table 2. OTUs of *Moina macrocopa* complex by three different species delimitation methods. *UTOs del complejo* Moina macrocopa *aplicando tres métodos diferentes para delimitar las especies.*

MPTP and ABGD analyses gave three groups instead of five as Barcode Identification request showed (Table 2). These three groups correspond to European clade (possibly the real *Moina macrocopa* (Straus, 1820), American clade *Moina americana* Goulden, 1968 n. stat. and the Asiatic clade (*Moina* cf. *macrocopa*). Differences in the number of groups delimited by BIN system and the other tools (MPTP and ABGD) five and three respectively, is due to the BIN system that is more sensitive than the other tools. It uses a threshold of maximum divergence of 2.2 % to delimitate the clusters and it should be mentioned that is continuously evolving in new BINs if more information is added (Ratnasingham & Hebert, 2007, 2013).

Kimura 2-parameters (K2P) mean intraspecific distances for each population in *Moina macrocopa* s.l. (European clade) are: 0.41 % in BOLD:ACH4664, 0 % in BOLD:AAK6825 and 0.75 % in BOLD:ACA1705. In the case of *Moina americana* Goulden, 1968 n. stat. (American Clade, BIN number BOLD:AAC3108), the mean intraspecific distance is 0.64 %. (Table 3). Interspecific distances ranged from 3.72 % to 13.36 %. The European clade showed the minimum mean interspecific distance between its populations (Russian population has not been considering for this analysis, due to the low number of available sequences in BOLD). The biggest mean interspecific distance 13.36 % is between the Asiatic clade (BOLD:ADF9261) evidenced by Bekker *et al.* (2016) and the European population with BIN number BOLD:ACA1705 that it is distributed in Hungary, Mexico and Russia.

The low intraspecific divergence showed by the American clade (BOLD:AAC3108) which includes sequences from *terra typica* in North America and other places from North and South America suggest the incipient expansion of this species. The high divergence in the European clade indicates that *Moina macrocopa* s.l. is possibly a species complex composed by at least three distinct species, two of them confirmed by

Table 3. K2P Mean Genetic divergences (intra and interspecific) of *Moina macrocopa* complex. Bold capitals: Mean Intraspecific distances. Abbreviations: H: Hungary, MX: Mexico, RS: Russia, SP: Spain, and CA: Canada. *Divergencias genéticas K2P medias (intra e interespecíficas) del complejo* Moina macrocopa. *Letra en negrita: Distancias intraespecíficas medias. Abreviaturas: H: Hungría, MX: México, RS: Rusia, SP: España y CA: Canadá.*

		Moina macrocopa European Clade		*Moina* cf *macrocopa* Asiatic Clade	*Moina americana* n. stat. American Clade
		H, MX, RS BOLD:ACH4664	SP, MX, RS BOLD:ACA1705	RS BOLD:ADF9261	CA, MX BOLD:AAC3108
Moina macrocopa European Clade	H, MX, RS BOLD:ACH4664	**0.41**			
	SP, MX, RS BOLD:ACA1705	3.72	**0.75**		
Moina cf *macrocopa* Asiatic Clade	RS BOLD:ADF9261	13.36	12.64	**0**	
Moina americana n. stat. American Clade	CA, MX BOLD:AAC3108	12.74	13.17	6.62	**0.64**

Table 4. Mean GC% content at the sequence composition of the COI Gene. *Contenido medio de GC% en la composición de las secuencias del gen COI.*

	European clade (36.3 ± 0.0826)			Asiatic clade	American Clade
BIN	ACH4664	AAK6825	ACA1705	ADF9261	AAC3108
Mean GC% content (± SE)	36.03 ± 0.0831	35.31 ± 0	36.59 ± 0.0496	34.97 ± 0.033	35.81 ± 0.092

morphology, genetics and distribution that corresponds to BIN numbers ACH4664 and ACA1705, the last one corresponding to the real *Moina macrocopa* (Straus, 1820). Finally, the high divergences between the clades from Europe and America corroborates our hypothesis that they are different species.

Regarding the GC content at the sequence composition (Table 4), it ranges from 34.97 to 36.59. Populations from cooler regions show low GC content than the other clades.

Neighbor-Joining and Maximum Likelihood analysis

ML analysis of COI gene is consistent with previous studies, showing that each clade is a monophyletic cluster. The branch of each cluster is highly robust (Fig. 4). We emphasize that the Id tree is not a complete phylogenetical analysis. Although it delimitates five groups, but we cannot ascertain in deep the relationships between these groups.

Genetic Diversity and haplotype analyses

Genetic diversity indexes and the results of neutrality test for COI are shown in Table 5. The average number of pairwise nucleotide differences (K), nucleotide diversity (p) and haplotype diversity (Hd) varies among the clades showing elevated levels of diversity inside the clades. A negative Tajima's D showed by the Asiatic clade demonstrates low levels of both low and high-frequency polymorphism. In total, 18 COI haplotypes were detected including four in the American clade (from two countries) (Fig. 5). The Asiatic clade included two haplotypes from

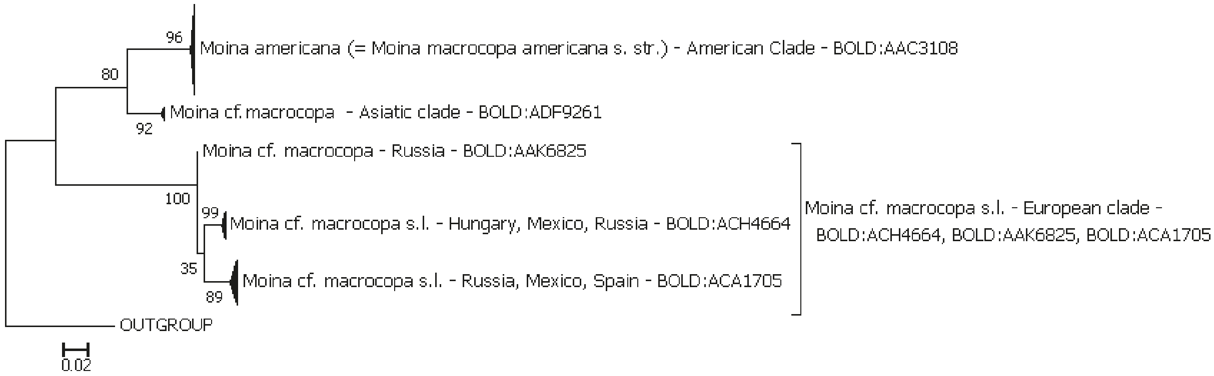

Figure 4. Id tree inferred by ML cluster analysis. Bootstrap values (500 replicates) are shown above the branches. The scale bar shows K2P distances. The node of each clade with multiples specimens is collapsed to a vertical triangle, with the horizontal depth indicating the level of intraclade divergence. *Árbol de identificación inferido a través del análisis de clusters de ML. Los valores de Bootstrap (500 réplicas) están sobre las ramas. La escala muestra las distancias K2P. El nodo de cada uno de los clados, está colapsado en un triángulo vertical, la profundidad horizontal indica el nivel divergencia dentro del clado.*

Sakhalin area (Russia), and the European clade contained eight haplotypes from four different regions, with the dominant haplotype, shared by Hungary and Mexico (Calderitas) and the American clade also shows eight haplotypes disseminated from Canada to Bolivia.

DISCUSSION

Due to the high number of characters, analyzed in this paper, (morphological, genetical divergences and geographical distribution), and the differences found between *Moina macrocopa* s.l. (Old World clade) and *Moina americana* Goulden, 1968 n. stat. (the American clade) and with the idea to clarify this group of species, we propose to elevate to species level the subspecies *Moina macrocopa americana* Goulden, 1868. So, we propose the name *Moina americana* Goulden, 1968 n. stat., with a BIN number BOLD:AAC3108 to facilitate its identification, in place of *Moina macrocopa americana* Goulden, 1968.

The detailed morphological studies such the Scanning microscope allowed verification of some dubious characters, as the “nucal pore” and the claw pecten, not clearly visible using traditional techniques. The “nucal pore” according to Alonso (1996) is one of the most significant characters of the species not seen previously in other congeners, and it did not appear in the descriptions of the species of Goulden (1968) and Elmoor-Loureiro *et al.* (2010). One other variable character of the species, the faint claw pecten, has also contributed to the confusion. Depending on the view of this setation on the claw, one might either conclude that a pecten is, or is not, present. This accounts for the different description of *Moina banffyi* (Daday, 1883) which was said to lack a pecten while *Moina esau* was described as having one (Brehm, 1936). Both forms are definitely the same species. In this study we confirm the presence of these characters in the *Moina macrocopa* complex species and we remark the importance of the “nucal pore” to identify the species along with the anterior seta on the penultimate segment of the female’s first trunk limb toothed. Differences found by Elmoor-Loureiro *et al.* (2010) between South American populations of *Moina macrocopa* s.l. must be confirmed by genetical analysis to describe the real identity of this *Moina* sp. In case of genetic characters, our results coincident with the morphology and the maximum divergence threshold of 3 % to delimitate the species level (Hebert *et al.*, 2003b; Bekker *et al.*, 2016). These results also agree with the morphological differences previously published (Straus, 1820; Arévalo, 1920; Goulden, 1968; Smirnov, 1976; Elías-Gutiérrez & Zamuriano-Claros, 1994; Alonso, 1996; Paggi, 1997; Elmoor-Loureiro *et* *al.*, 2010) between the American and Old World morphotypes, and add some taxonomical remarks that should be considered for further descriptions of new species belonging to these species complexes. Specimens with genetic divergence lower than 3 % do not present morphological differences, so we can conclude that BIN number-AAC3108, representing *Moina americana* Goulden, 1968 n. stat. is present in Canada, USA (from *terra typica*, near the original type locality, see specimens with Process ID: BCRUS111-10, BCRUS107-10, and BCRUS106-10 in BOLD), and is distributed in Mexico and Bolivia. The low variability of the American species, the high number of haplotypes (8 haplotypes) and the broad geographical area of distribution, shown by our results, indicates that this species is beginning an expansion that started less than 250 000 years ago (according to Ratnasingham & Hebert, 2007).

The high existent divergence between the populations in the Old World can be interpreted as a longer time of separation represented by the BIN numbers ACH4664 andACA1705 with a maximum divergence of 3.72 %. This divergence is also coincident with morphological traits as mentioned above.

The use of molecular markers helped us to delimitate species, but the sole use of these tools, will cause the loss of essential information (Will *et al.*, 2005). The phenotypical plasticity present in different geographical areas for invertebrate evidence high cryptic speciation and not morphotypes or cosmopolitanism, as it has been considered (Alcántara-Rodríguez *et al.*, 2012; Karanovic 2015; Lavinia *et al.*, 2017).

In other regard, haplotypes shared between distant geographical sites evidence a recent translocation of both species, *Moina macrocopa* s.l. and *Moina americana* Goulden, 1968 n. stat., possibly due to human activities such as it has been documented several times for the European clade of this species and for other microinvertebrate taxa (Elías-Gutiérrez & Zamuriano- Claros, 1994; Miracle *et al.*, 2013; Vignatti *et al.*, 2013; Montoliu *et al.*, 2015) and also, due to biotic (birds) or/and abiotic factors. It is widely documented that cladocerans during the glaciations of Pleistocene survived in small isolated refugia during the Ice Age. Since the thaw, they colonized many new areas due to the ephippia stuck in the legs of birds (Adamowicz *et al.*, 2002; Korovchinsky, 2006), and given their opportunistic and potentially invasive nature (Vignatti *et al.*, 2013) they became common in new habitats or environments. In this regard, climate change drives birds to change their migration routes, and with them,

Table 5. Genetic diversity index and neutrality test (Fu & Tajima's D) on the mitochondrial COI sequences of *Moina macrocopa* complex. *n*: number of sequences; S: number of polymorphic sites; h: number of haplotypes; Hd: haplotype diversity; p: nucleotide diversity; k: average number of pairwise nucleotide differences. Tajima's D: A negative Tajima's D signifies an excess of low frequency polymorphisms relative to expectation. A positive Tajima's D signifies low levels of both low and high frequency polymorphisms. Statistical significance: Not significant, *P* > 0.1. *Índices de diversidad genética y test de neutralidad (Fu y Tajima's D) de las secuencias del gen mitocondrial COI del complejo* Moina macrocopa. n*: número de secuencias; S: número de sitios polimórficos; h: número de haplotipos; Hd: diversidad haplotípica; p: diversidad nucleotídica; k: número promedio de diferencias de nucleótidos por pares. Tajima's D: un valor negativo en el test significa un exceso de polimorfismos de baja frecuencia en relación con las expectativas un valor positivo en el test significa bajos niveles de polimorfismos de baja y alta frecuencia. Significancia estadística: No significante,* P > *0.1.*

	n	**S**	***h***	**Hd**	**π**	**K**	**Tajima's D (Significance)**		**Fu &Li (Significance)**	
EUROPE	28	19	8	0.86	0.02506	7.57	1.57	*P* > 0.10	0.96	*P* > 0.10
ASIA	4	1	2	0.5	0.00166	0.5	-0.5	*P* > 0.10	1.44	*P* > 0.10
AMERICA	21	6	8	0.855	0.00760	2.286	0.18769	*P* >0.10	0.18769	*P* >0.10
TOTAL	64	59	18	0.937	0.07934	24.04	2.28	*P* < 0.5		

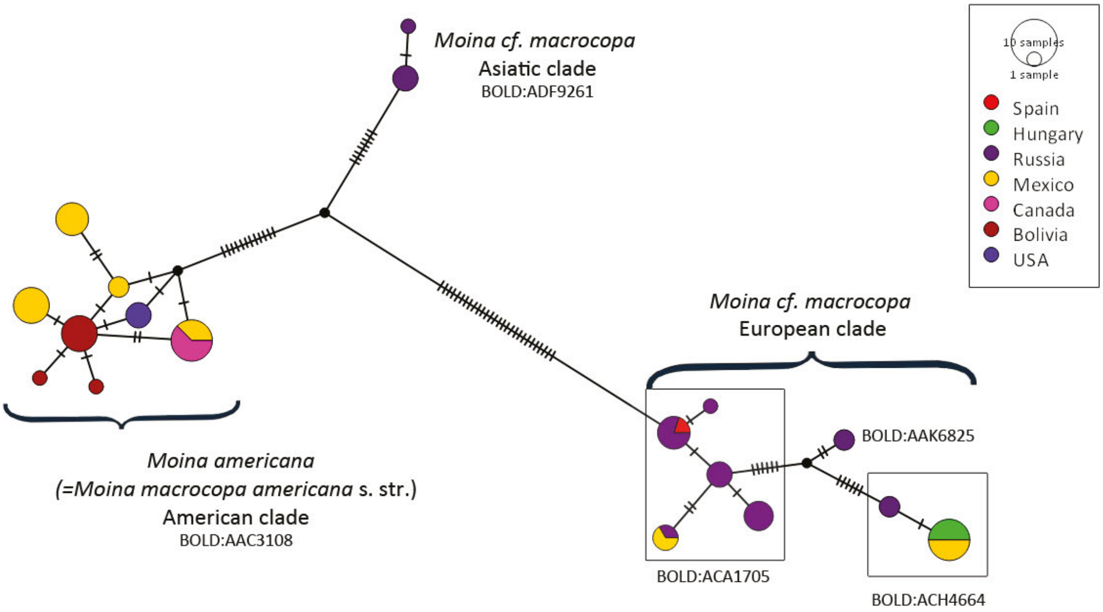

Figure 5. COI Haplotype network of *Moina macrocopa* complex. Each circle indicates a unique haplotype and variation in circle reflects the number of sequences assigned to haplotypes. Colors represent the countries of each haplotype. *Red de haplotipos del gen COI del complejo* Moina macrocopa*. Cada círculo indica un haplotipo único y la variación en el tamaño del círculo refleja el número de secuencias asignadas a cada haplotipo. Los colores representan los países en donde se encuentra cada haplotipo.*

Moina and other invertebrates will benefit to disperse to new warm regions, where before it was not possible to survive. Remark that the type locality of *Moina americana* n. stat., Cheyenne Bottoms, is the largest marsh in the interior of the United States, deeply modified by man, was designated a Wetland of International Importance in 1988 by the Ramsar Convention on Wetlands. The area is considered the most important shorebird migration point in the western hemisphere. Approximately 45 percent of the North American shorebird population stops at the Bottoms during spring migration so, it will be probably the reason to find the same haplotype of *Moina americana* n. stat. distributed all over the American continent.

The proximity existent (morphological, genetical and geographical) between the different populations of the American clade and the lack of interest in this American species (because the European species is used as bioindicator and food for aquaculture) suggest that their distribution can be due to biotic or abiotic factors and not by an anthropogenic translocation.

Nevertheless, the Old World species found in Calderitas (Mexico) seems to be a recent human mediated translocation owing to the little divergence intraspecific existent between the Mexican populations and the Europeans (ACH4664: 0.41 % and ACA1705: 0.75 %). We hypothesize that a European student of Amphibia, who surveyed the ponds before us, introduced the resistant ephippia inadvertently with his net in search of tadpoles. Both species were found inhabiting in sympatry in two close, isolated temporary pools, with no fish. Otherwise, there is no explanation, because *Moina macrocopa* s.l. was included in a list of 94 potential invaders of inland waters, coastal and littoral lakes in Mexico (Okolodkov *et al.*, 2007), but it has not been previously found in the tropics (Elías-Gutiérrez *et al.*, 2001; Elías-Gutiérrez *et al.*, 2008a; Elías-Gutiérrez *et al.*, 2008b).

In this work, we increased the distributional area of *Moina americana* n. stat. Its new range is from 58.77 °N to 18.24 °S, but nowadays, only restricted to the American continent instead of just at the USA as Goulden (1968) stated.

Moina americana Goulden, 1968 n. stat. seems to prefer permanent water bodies, highly eutrophic and with an excess of food supply as Los Gringos dam and Texcoco Lake.

The use of an integrative approach to delimitate the species present in an ecosystem will facilitate the creation of zooplankton baselines that will permit more accurate biomonitoring and the early detection of changes in the environment.

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Effect of a cyanobacterial diet on the competition between rotifers: a case study in Lake Albufera of Valencia, Spain

S.S.S. Sarma[1], S. Nandini[1], M.R. Miracle† and E. Vicente[2,*]

[1] Laboratorio de Zoología Acuática, División de Investigación y Posgrado, Universidad Nacional Autónoma de México, Campus Iztacala, Av. de los Barrios #1, Los Reyes, Iztacala, Tlalnepantla, CP 54090. Estado de México, Mexico.
[2] Dept. Microbiologia i Ecologia & ICBiBE, Universitat de València, 46100-Burjassot (Valencia), Spain.
† Deceased.

* Corresponding author: eduardo.vicente@uv.es

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ABSTRACT

Effect of a cyanobacterial diet on the competition between rotifers: a case study in Lake Albufera of Valencia, Spain

Brachionus havanaensis is predominantly distributed in the Nearctic and Neotropical regions. It is now found even in the Palearctic and Oriental regions. During the summer of 2015 we found high densities (> 500 ind./l) of this species and low numbers of *Brachionus angularis* in Lake Albufera where the cyanobacterium (*Microcystis aeruginosa*) was dominant in the phytoplankton community. We hypothesized that the cyanobacterium was toxic to *B. angularis* but not to *B. havanaensis* hence the latter would outcompete the former in the presence of this cyanobacterium. To test the hypothesis, we conducted competition experiments between two coexisting rotifer species (*B. angularis* and *B. havanaensis*) from the Lake Albufera of Valencia using as food the green alga (*Nannochloropsis oculata*) and sonicated colonies of *M. aeruginosa* (single cells), separately and together (1:1 ratio based on biomass). The competition experiments using the population growth approach showed that *B. havanaensis* in controls had higher population abundances than *B. angularis*. However, when grown both together, *B. havanaensis* had a reduced population growth (nearly 50 %) in relation to *B. angularis*, in the absence of *Microcystis* in the diet. This reduced growth was more than that in the treatment of *B. havanaensis* alone when grown on a mixed diet of *Microcystis* and *Nannochloropsis oculata*. *Brachionus havanaensis* in all treatments (alone or in the presence of *B. angularis*) died completely when cultured on 100 % *M. aeruginosa*. The population growth rates of *B. angularis*, alone or in competition with *B. havanaensis*, were similar when *Microcystis* was excluded from the diet. However, *Microcystis* alone or together with *Nannochloropsis* was highly detrimental for the population growth of *B. angularis*. Our results showed the differences in the competitive outcome between the rotifers depending on the presence or absence of toxic cyanobacteria in the diet. Our study also showed that an exclusive diet of this cyanobacterium was toxic to both rotifers.

Key words: *Brachionus*, Rotifera, Population dynamics, *Microcystis*, *Nannochloropsis*

RESUMEN

Efecto de la dieta con cianobacterias en la competencia entre rotíferos: un caso de estudio en la laguna de la Albufera de Valencia, España

Brachionus havanaensis *se distribuye predominantemente en las regiones Neártica y Neotropical. Ahora se encuentra incluso en las regiones Paleártica y Oriental. Durante el verano de 2015, encontramos altas densidades (> 500 ind./l) de esta especie y bajas abundancias de* Brachionus angularis *en la laguna de la Albufera, donde la cianobacteria (*Microcystis aeruginosa*) fue dominante en la comunidad de fitoplancton. Nuestra hipótesis fue que la cianobacteria era tóxica para* B. angularis *pero no para* B. havanaensis*, por lo que este último superaría al primero en presencia de dicha cianobacteria. Para probar la hipótesis, realizamos experimentos de competencia entre estas dos especies de rotíferos (*B. angularis *y* B. havanaensis*) que coexisten en la laguna de la Albufera de Valencia utilizando como alimento el alga verde (*Nannochloropsis oculata*) y colonias sonicadas de* M. aeruginosa *(células individuales) por separado y juntas (en relación 1:1 basada en biomasa). Los experimentos de competencia basados en el seguimiento del crecimiento poblacional mostraron que en los controles* B. havanaensis *conseguía* *un mayor crecimiento poblacional que* B. angularis *cuando lo hacían separadamente. Sin embargo, cultivándolos ambos juntos,* B. havanaensis *tuvo una reducción del crecimiento poblacional (casi 50 %), con respecto a* B. angularis*, en ausencia de* Microcystis *en la dieta. Esta reducción del crecimiento fue mayor que en el tratamiento de* B. havanaensis *individualmente, cuando se cultivó con una dieta mixta de* Microcystis *y* Nannochloropsis oculata*.* Brachionus havanaensis *en todos los tratamientos (solos o en presencia de* B. angularis*) murió completamente cuando se cultivó en 100 % de* M. aeruginosa*. Las tasas de crecimiento poblacional de* B. angularis*, solos o en competencia con* B. havanaensis*, fueron similares cuando se excluyó a* Microcystis *de la dieta. Sin embargo, el cultivo solo con* Microcystis *o junto con* Nannochloropsis *fue altamente perjudicial para el crecimiento poblacional de* B. angularis*. Nuestros resultados mostraron el diferente resultado de los experimentos de competencia entre estos dos rotíferos en función de la presencia o ausencia de cianobacterias tóxicas en la dieta. Nuestro estudio también mostró que una dieta exclusiva de esta cianobacteria era tóxica para ambas especies de rotíferos.*

Palabras clave: Brachionus*, Rotifera, dinámica poblacional,* Microcystis*,* Nannochloropsis

INTRODUCTION

Competition in aquatic ecosystems is an important natural process that structures the plankton composition (Vanni, 1986). Studies on the competitive interactions within the zooplanktonic groups are usually conducted using large species such as cladocerans (Smith & Cooper, 1982), which differ from the competitive outcome among the smaller sized zooplankton such as rotifers (Gilbert, 1985). For example, cladocerans are capable of breaking and then feeding on toxic filamentous cyanobacteria which rotifers are incapable of (Sikora & Dawidowicz, 2017). Therefore, often due to the mechanical limitation of consuming cyanobacteria, rotifers are incapable of competing with cladocerans. Even when the toxic cyanobacterial colonies are sonicated to single cells and offered as a diet to competing rotifers and cladocerans, the latter usually dominate due to their higher food gathering ability and the mechanical damage (from the powerful swimming currents) they inflict to the rotifer eggs (MacIsaac & Gilbert, 1989). Therefore, it becomes evident that when unequally sized zooplankton groups compete, the smaller groups become competitively inferior especially under higher food levels (Sarma *et al.*, 1996).

When two similar sized zooplankton species compete for food, the outcome depends on many different factors including the relative feeding and filtration rates, the magnitude of population growth rate and initial densities of the competing species (Sarma *et al.*, 1999, Nandini *et al.*, 2007). Also, if the medium contains toxic cyanobacteria, then the relative tolerances of competing species to the toxins are also crucial in deciding the outcome (Alva-Martínez *et al.*, 2007). For example, when two similar sized rotifers, *Brachionus havanaensis* and *Brachionus calyciflorus* were cultured together on *Microcystis* mixed with green alga at different proportions we observed a more adverse effect on *B. calyciflorus* than on *B. havanaensis* (Alva-Martínez *et al.*, 2009). However, if the competing species have similar sensitivities to toxins, then the competitive outcome is independent of the toxic cyanobacteria. For example, Lin *et al.* (2014) have shown that two rotifers of the family Brachionidae, *Plationus patulus* and *Brachionus havanaensis* had similar sensitivities to sonicated cells of a toxic strain of *Anabaena* sp. Both species failed to grow in the presences of this cyanobacterium, and thus competition was not influenced by the toxins. Therefore, although cyanobacterial toxicity influences zooplankton growth rates, it is not necessarily the deciding biotic factor that determines the competitive outcome among zooplankton species.

Lake Albufera of Valencia is a shallow hypertrophic waterbody on the Mediterranean Coast of Spain. The plankton of this waterbody has been extensively studied over three decades (Vicente & Miracle, 1992; Romo *et al.*, 2008). The phytoplankton is composed of toxic colonial cyanobacteria, mainly *Microcystis aeruginosa*, while the rotifers are dominated by the genus *Brachionus*. Among the species of *Brachionus*, *B. havanaensis* is common in American epicontinental waters (Segers, 2008). Its natural abundances can be as high as 1000 ind./l (Nandini *et al.*, 2016). It has the ability to reach extremely high population abundances (up to 600 ind./ml, under experimental conditions (Pavón-Meza *et al.*, 2004) and to resist toxic effects of the colonial cyanobacteria including *Microcystis* spp. Though its occurrence has been reported in temperate European water-bodies (Segers, 2008), it is generally found in low abundances (< 50 ind./l). However, during the summer 2015, we found very high densities of *B. havanaensis* (> 500 ind./l) but low abundances of another brachionid rotifer *B. angularis* in Lake Albufera. The phytoplankton of the lake at that time contained blooms of *Microcystis aeruginosa*. Therefore we hypothesized that low abundances of *B. angularis* were due to the combined effect of competitive pressure from *B. havanaensis* and the toxic effects of *M. aeruginosa*.

The aim of the study was, therefore, to test which of the two natural forces, competition from congeners or toxicity from *Microcystis* had a higher impact on the population growth of *B. havanaensis* and *B. angularis*.

MATERIAL AND METHODS

Zooplankton samples were collected using a plankton net (pore size 50 µm) from Lake Albufera by filtering about 50 L of water. The phytoplankton, dominated by *M. aeruginosa*, was separately (100 L) collected using a plankton net and stored in the dark at 4 °C in a temperature-controlled chamber. From the live zooplankton samples we isolated a few individuals of *Brachionus havanaensis* and *Brachionus angularis* using stereomicroscope in the laboratory. From these, we established a clonal culture for each rotifer starting with a single female. The rotifers were fed the green alga *Nannochloropsis oculata* at a density of 0.5 x 10^6 cells/ml and maintained in reconstituted moderately hard water (EPA medium). This medium was prepared by dissolving 96 mg $NaHCO_3$, 60 mg $CaSO_4$, 60 mg $MgSO_4$ and 4 mg KCl in one litre of distilled water (Weber, 1993). *Nannochloropsis oculata* was axenically batch-cultured in 2 L glass flasks using Bold's medium (Borowitzka & Borowitzka, 1988). When the alga reached the log phase (after seven days following inoculation at a density of 0.1 x 10^6 cells/ml), it was harvested and concentrated by centrifugation at 3000 rpm for 5 min. The concentrated alga was rinsed and re-suspended in a small volume (5 ml) of distilled water. The density of the harvested alga was estimated using a Neubauer haemocytometer.

For obtaining the sonicated cells of *M. aeruginosa*, we harvested the floating colonial cyanobacteria from the cold-chamber by syphoning using a Pasteur pipette. Later, the concentrated colonial *M. aeruginosa* was filtered using different meshes to remove large zooplanktonic organisms and smaller phytoplankton cells. The remaining cyanobacterial composition showed more than 93 % *M. aeruginosa* which was sonicated for four minutes at 20 kHz. Microscopic observations of the sonicated cells revealed that more than 90 % of the biomass was in single-celled form of *M. aeruginosa*. The individual cell size of *M. aeruginosa* and that of *Nannochloropsis oculata* was similar (4-5 µm). However, we used the biomass equivalent of *M. aeruginosa*, *Nannochloropsis oculata* or their mixed diet for rotifers in the test jars.

The population growth experiments for both rotifer species were conducted simultaneously. For the experiments, we used 40 ml glass recipients as test jars, and each jar contained 30 ml EPA medium with one of the chosen diet type and combination. In all we used 36 test jars: *B. havanaensis* alone, *B. angularis* alone and together (competition) = 3 x food types *N. oculata* alone, *M. aeruginosa* alone and their mixed diet = 3 x 4 replicates for each treatment. Thus, into each test jars containing the chosen diet at the density of 0.5 x 10^6 cells/ml, we individually introduced one of the two rotifer species at an initial density of 1 ind./ml (or both at a ratio of 1:1). The initial composition of rotifers in the test jars consisted of 80 % neonates and 20 % adults. The test jars were maintained in a temperature-controlled chamber set at 22±0.1 °C, pH 7.0–7.4, and with photoperiod light-dark of 16-8 hours diffuse fluorescent illumination.

Following initiation of growth experiments, after every 24h we counted the number of live rotifers in each jar, initially by total count, and as the density of the population increased to 50 ind./ml in some replicates, we used two aliquots of 1 ml each. Following quantification of the rotifers density, we replaced them into a freshly prepared medium with an appropriate algal-cyanobacterial composition.

Based on the data collected, we derived the rate of population increase (r) per day using the exponential growth equation: $r = (ln\ N_t - ln\ N_0)/t$, where N_0 = initial rotifer density; N_t = rotifer density after time t; and t = time in days. In treatments where a peak of population abundance was not evident, the growth rate was calculated from the slope between $ln\ N$ and time (Sibly & Hone, 2002) and when growth did not occur, the r was not derived.

The peak population density and the rate of population increase for each rotifer species were statistically treated using two-way ANOVA. For multiple comparisons, we used a post hoc (Tukey) test (SigmaPlot ver. 12).

RESULTS

Population growth curves of *Brachionus havanaensis* cultured separately and together with *B. angularis* fed *Nannochloropsis oculata* (100 %), sonicated *Microcystis aeruginosa* (100 %) or on the mixed diet (1:1 ratio of *N. oculata*: *M. aeruginosa*) differed depending on the treatment (Fig. 1). When cultured on *N. oculata*, in the presence or absence of competition from *B. angu-*

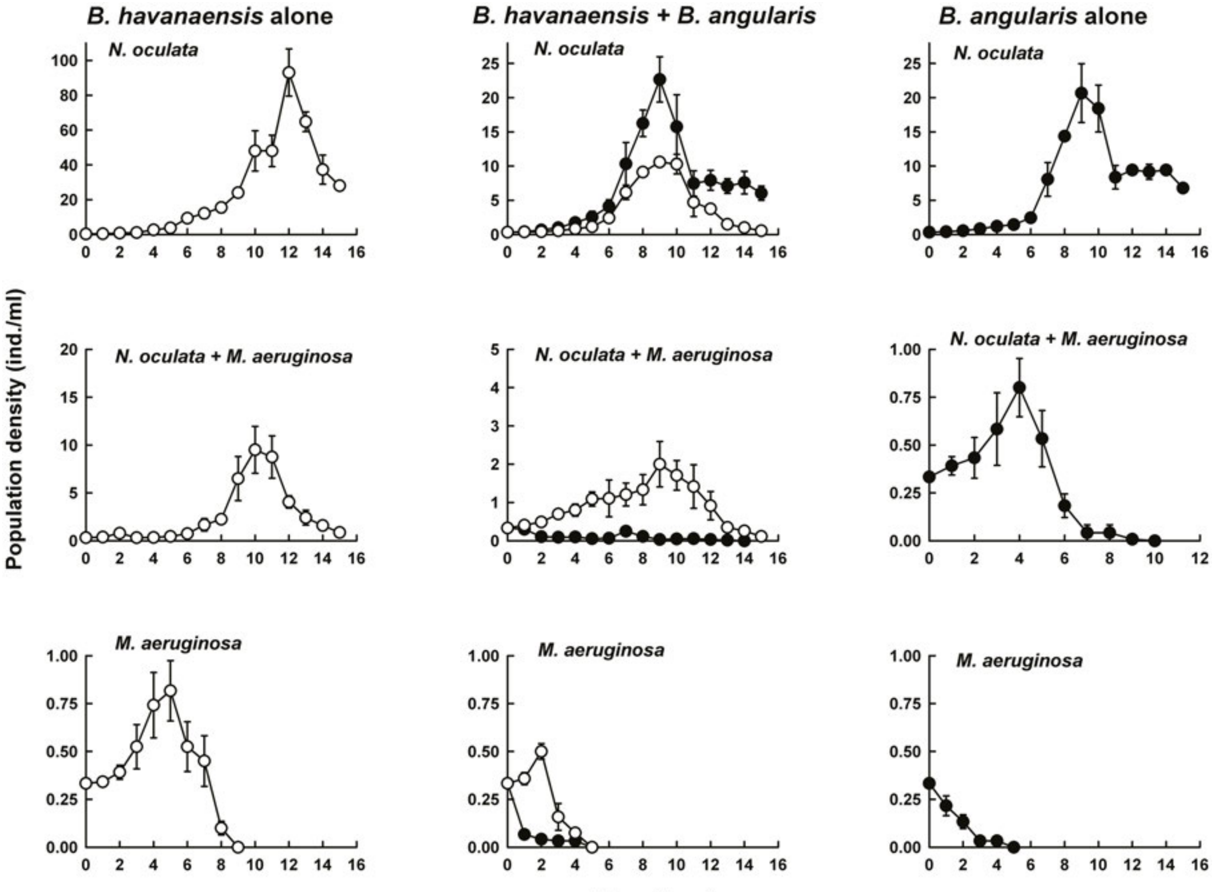

Figure 1. Population growth curves of *Brachionus havanaensis* and *B. angularis* cultured alone or together on *Nannochloropsis oculata*, sonicated cells of *Microcystis aeruginosa* or on the mixed diet. Shown are the mean±standard errors based on four replicates. Note the differences in the scale on Y-axis. Open circle: *B. havanaensis*; closed circle: *B. angularis*. *Curvas de crecimiento poblacional de* Brachionus havanaensis *y* B. angularis *cultivadas solas o juntas, usando* Nannochloropsis oculata*, células sonicadas de* Microcystis aeruginosa *o en la dieta mixta. Se muestran el promedio ± errores estándar basados en cuatro repeticiones. Nótese las diferencias en la escala en el eje Y. Círculo abierto:* B. havanaensis*; Círculo cerrado:* B. angularis.

laris, *B. havanaensis* showed an initial lag phase of about four days, an exponential phase of about seven days and after that the population began to decline. With the inclusion of sonicated *M. aeruginosa* in the diet, *B. havanaensis* had a longer lag phase (6 days). On a diet of *M. aeruginosa* alone, *B. havanaensis* grew until 6 days and then declined but on this diet and the presence of the competitor the decline began after 3 days. The growth curves of *B. angularis* cultured on a diet of *N. oculata*, alone or in the presence of a competitor were similar. However, with the inclusion of *M. aeruginosa*, the population of *B. angularis* hardly grew beyond the inoculation density and when fed on an exclusive diet of *M. aeruginosa*, the populations of *B. angularis* completely collapsed by the fifth day.

Depending on the treatment and the test rotifer species, the peak population abundances of *B. havanaensis* and *B. angularis* varied from 1-95 and 1-23 ind./ml, respectively. The presence of a competitor or the toxic diet had differential effects on the maximal population densities achieved by the tested brachionid rotifers. For example, for *B. havanaensis*, the presence of *M. aeruginosa* (in the mixed diet) or the presence of competitor (in the absence of cyanobacteria) had a similar adverse effect on the peak population density. On the other hand, for *B. angularis* in the absence of *M. aeruginosa*, the presence of a competitor, *B. havanaensis*, had no adverse effect on the peak population densities.

The rates of population increase of *B. havanaensis* and *B. angularis* in treatments containing only *N. oculata* but without competition were 0.40 and 0.30 /d, respectively. When the mixed diet (*Nannochloropsis* and *Microcystis*) was offered as food, the growth rates of *B. angularis*, but not *B. havanaensis*, became negative. For either rotifer species, 100 % *M. aeruginosa* did not permit sustained populations, and hence the derivation of growth rates was not feasible (Table 1).

Statistically, the peak population density of *B. havanaensis* was significantly influenced by the diet type, competition and their interaction ($p <$

Table 1. Data on the peak population abundance (ind./ml) and the rate of population increase (r) per day of *B. havanaensis* and *B. angularis* cultured separately and together in the presence (N+M or M) and absence (N) of sonicated cells of *M. aeruginosa*. For each rotifer species data carrying the same alphabet are not significant ($p > 0.05$, Tukey test). N = *Nannochloropsis oculata*, M = *Microcystis aeruginosa*; N+M = *N. oculata* + *M. aeruginosa* (50 % each). *Datos sobre la abundancia máxima de la población (ind./ml) y la tasa de aumento de la población (r) diaria de* B. havanaensis *y* B. angularis *cultivados por separado y juntos en presencia (N+M o M) y ausencia (N) de células sonicadas de* M. aeruginosa. *Para cada especie de rotíferos los daots que llevan la misma letra no son significativos (*p *> 0.05, test de Tukey). N =* Nannochloropsis oculata*, M =* Microcystis aeruginosa*; N+M =* N. oculata + M. aeruginosa *(al 50 % cada una).*

Rotifer species	Test conditions / Treatment					
	Without competition			With competition		
	N	N+M	M	N	N+M	M
	Peak population density (ind./ml)					
B. havanaensis	95±12^a	10±2^{b,d}	1±0^c	11±1^d	2±1^c	1±0^c
B. angularis	21±3^a	1±1^b	1±0^b	23±3^a	1±1^b	1±0^b
	Rate of population increase per day					
B. havanaensis	0.403± 0.031^a	0.174± 0.020^b	-	0.252± 0.017^c	0.078± 0.017^d	-
B. angularis	0.302± 0.011^a	-0.076± 0.019^b	-	0.233± 0.031^a	-0.146± 0.010^b	-

0.001 two-way ANOVA). However, for *B. angularis*, it was only the diet type that had a significant effect on the peak population abundances; neither the presence of competitor nor the interaction of competitor x diet had any significant impact on this parameter ($p < 0.05$). The rate of population increase of both the rotifer species was significantly affected ($p < 0.01$) by the diet type and the competition, but the interaction was not ($p > 0.05$, Table 2).

DISCUSSION

The dominance of the typical American planktonic rotifer species, *B. havanaensis*, in the zooplankton of Lake Albufera suggests that it has an invasive tendency (Devin & Beisel, 2007). It is often found in waterbodies dominated by toxic cyanobacteria (Nandini *et al.*, 2005). Zooplankton composition of Lake Albufera over a three-decade period indicates the presence of

Table 2. Results of the two-way ANOVA conducted for the peak population density and the rate of population increase per day of *Brachionus havanaensis* (Bh) and *Brachionus angularis* (Ba) cultured separately or together (Factor A: competition) using *Nannochloropsis oculata*, *Microcystis aeruginosa* or on their mixed diet (Factor B: diet types). *Resultados del ANOVA bidireccional realizado para la densidad máxima de población y la tasa de aumento de población por día de* Brachionus havanaensis *(Bh) y* Brachionus angularis *(Ba) cultivados por separado o juntos (Factor a: competición) usando* Nannochloropsis oculata*,* Microcystis aeruginosa *o su mezcla como dieta (Factor B: tipos de dieta).*

Source of Variation	DF	SS	MS	F	***P***
Peak population abundance					
Bh					
Competition (A)	1	5645.734	5645.734	60.596	<0.001
Diet (B)	2	13603.104	6801.552	73.002	<0.001
Interaction of A X B	2	8685.165	4342.582	46.609	<0.001
Error	18	1677.059	93.170		
Ba					
Competition (A)	1	2.100	2.100	0.174	0.681
Diet (B)	2	2492.614	1246.307	103.330	<0.001
Interaction of A X B	2	7.941	3.970	0.329	0.724
Error	18	217.099	12.061		
Rate of population increase					
Bh					
Competition (A)	1	0.061	0.061	31.040	<0.001
Diet (B)	1	0.162	0.162	82.634	<0.001
Interaction of A X B	1	0.003	0.003	1.574	0.234
Error	12	0.024	0.002		
Ba					
Competition (A)	1	0.019	0.019	12.297	0.004
Diet (B)	1	0.573	0.573	368.518	<0.001
Interaction of A X B	1	0.000	0.000	0.001	0.992
Error	12	0.019	0.002		

rotifer genera such as *Keratella*, *Polyarthra* and *Anuraeopsis*. Though several members of the genus *Brachionus* are commonly reported in this waterbody (Oltra *et al.*, 2001), *B. havanaensis* was not observed from the extensive zooplankton collections carried out during 2010-2011 (Sarma *et al.*, 2019) suggesting that this species was recently established in the Lake Albufera. *Brachionus havanaensis* is predominantly distributed in the Nearctic and Neotropical regions. Later, it was introduced into the Palearctic and Oriental regions (Segers, 2008). During the summer of 2015, we encountered much higher abundances of this species suggesting its ability to outcompete other members of *Brachionus* in this waterbody. Field collections from Mexican waterbodies too indicate its occurrence in relatively higher densities, ca. 1000 ind./l during certain months (Nandini *et al.*, 2016). There appear to be several factors favouring its dominance in different waterbodies: these include relatively higher resistance to natural toxins including cyanotoxins, higher population growth rates (Pavón-Meza *et al.*, 2004, 2008) and non-preferred prey item for invertebrate predators such as larval insects and copepods (Enríquez-García *et al.*, 2013).

Alva-Martínez *et al.* (2009) have shown that on a mixed diet consisting of a green alga and toxic *Microcystis aeruginosa*, the population of *Brachionus calyciflorus* crashed with increasing proportion of cyanobacteria in the diet, while under similar conditions, *B. havanaensis* showed consistently higher growth rates. Also, whether grown separately or together with *B. calyciflorus*, *B. havanaensis* was always numerically more abundant than the other competing brachionid rotifers regardless of the proportion of *Microcystis* in the diet. This confirms the resistance of *B. havanaensis* to *M. aeruginosa* over other brachionids. This is similar to our observations in this work where in the absence of competition when *B. havanaensis* was cultured on a mixed diet of *Nannochloropsis* and *Microcystis*, the population density was reduced to one-tenth; under comparable conditions, the population of *B. angularis* was reduced to one-twentieth. González-Pérez *et al.* (2018) have also documented that *B. havanaensis* is more resistant for two generations than another rotifer from the same family Brachionidae, *Plationus patulus*, when exposed to triclosan, an emerging contaminant in freshwater ecosystems. In the presence of strong invertebrate predators such as cyclopoids, *B. havanaensis* increases its growth rate than in the presence of less rapacious taxa such as turbellarians (Nandini *et al.*, 2014). Although adult copepods such as *Acanthocyclops*, consume *B. havanaensis* (García *et al.*, 2011), it is not a preferred item for many invertebrate predators (Nandini *et al.*, 2003) possibly due to long posterior spines (Garza-Mouriño *et al.*, 2005). In Lake Albufera, cyclopoids such as *Acanthocyclops americanus* are important predators that feed on rotifers including brachionids.

Competition for food between two (e.g., Sarma *et al.*, 1999) or more (e.g., Fernández-Araiza *et al.*, 2005) brachionid rotifers has received considerable attention since the seminal works on *Brachionus rubens* and *B. calyciflorus* by Rothhaupt (1988). These studies have shown that both biotic (e.g., food level, inoculation density) and abiotic (e.g., salinity, temperature) factors as well as species-specific characters (e.g., body size, intrinsic rate of population increase) influence the competitive outcome. Also, the presence of toxic substances, both xenobiotic (e.g., pharmaceuticals) and natural (e.g., cyanotoxins, salinity) are also known to affect the competition between two or more zooplankton species (Rebolledo *et al.*, 2018). In the present work, the competitive outcome between *B. havanaensis* and *B. angularis* varied depending on the presence or absence of *M. aeruginosa*. In the absence of cyanobacteria, *B. angularis* had an adverse effect on *B. havanaensis* by reducing its population densities by about 90 % and growth rates by about 65 %. On the other hand, under comparable conditions, *B. havanaensis* was able to affect adversely (by about 20 %) the growth rates but not the population abundances of *B. angularis*. However, in the presence of toxic *Microcystis* mixed with *Nannochloropsis*, the population of *B. angularis* completely crashed (r being negative) before termination of the experiments, but under similar conditions, *B. havanaensis* was able to maintain its population with positive growth rates. These results suggest that higher abundances of *B. havanaensis* in Lake Albufera were not possibly due to its competitive superiority over *B. angularis* but the interference of toxicity from *M. aeruginosa* which favoured *B. havanaensis*. It is important to note that the toxic effects of *M. aeruginosa* on the responses of the population level responses of both *B. angularis* and *B. havanaensis* are possibly modulated by the presence of the green alga (Paerl, 2018). This is evident in mixed diet treatments containing the green alga which possibly minimized the adverse effects of *Microcystis*. The fact that both rotifer species died within a week when exposed to an exclusive diet of *M. aeruginosa* suggests that this strain of cyanobacterium was indeed toxic. This is further confirmed from previous studies from this waterbody where the microcystin concentration in the lake was as high as 16 µg/l (Romo *et al.*, 2008) and the inclusion of sonicated cells of *M. aeruginosa* in the diet significantly reduced the growth rates of the cladocerans (Nandini *et al.*, 2017).

The peak population densities (up to 90 ind./ml) observed here for *B. havanaensis* are not uncommon for this species under laboratory conditions. For example, Pavón-Meza *et al.* (2004) observed that *B. havanaensis* is capable of reaching extremely high densities (up to 600 ind./ml) when fed on the green alga *Chlorella*. We were able to grow *B. angularis* to peak densities of about 20 ind./ml. Miracle *et al.* (2014) have cultured *B. angularis* isolated from Lake Albufera and fed on different size fractions of lake seston (0-15 µm particle size). They obtained peak population densities of about 20 ind./ml of *B. angularis*, similar to our observations in this study. The rate of population increase is yet another significant parameter often considered for interpreting the competitive outcome among different zooplankton species. For example, when *Anuraeopsis fissa* and *B. calyciflorus* were cultured together, the ability of the latter to replace the former was higher due to its higher population growth rates ($r = 1.5$ per day) than that of *A. fissa* ($r = 0.9$ per day) (Sarma *et al.*, 1996). In the present work, the r of *B. havanaensis* varied from 0.25 to 0.40/d depending on the presence or the absence of the competitor. Pavón-Meza *et al.* (2004) reported the r for this species in the range of 0.02 to 0.29 per day. For the strain of *B. havanaensis* we used here the r was slightly higher, but within the range reported for *Brachionus* (0.12 to 3.7 per day, Miracle & Serra, 1989). When *Nannochloropsis* alone was used as a diet, the r of *B. angularis* varied from 0.23 to 0.30 per day which is within the range (0.07 - 0.35 per day) known for this species (Walz, 1987).

The present work suggests the possible adverse effects of the exotic *B. havanaensis* on locally occurring brachionid rotifers in interacting with cyanobacterial blooms, common in the lake Albufera (Vicente & Miracle, 1992). *Brachionus havanaensis*, being pantropical (Segers, 2008), may easily colonize temperate waterbodies with the future projections of global warming (Hays *et al.*, 2005). Increase in temperature also favours bloom formation in *Microcystis*. Thus, both these factors together may adversely impact locally adapted brachionids in freshwater ecosystems of temperate regions.

CONCLUSIONS

An increase in global trade and transport has increased the mobility of organisms and broken down ecological barriers to the transport of species around the world. Rotifers, in particular, are easily transported and established across the world (Duggan, 2010). The ability of *B. havanaensis* to withstand competition and predation pressure and grow on diets such as cyanobacteria indicates that it can be a successful invasive species and can survive in regions outside its native continent (Koste, 1978). Although originally reported from the Nearctic and neotropical regions, it is now found in Oriental and Palearctic regions (Segers, 2008). It has, however been suggested that the presence of the same species in different parts of the world could be a case of cryptic speciation as was the case in *Brachionus plicatilis* (Pociecha *et al.*, 2007) and perhaps *B. havanaensis* is part of a species complex.

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Zooplankton as ecological quality indicator

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A comparative study of four indexes based on zooplankton as trophic state indicators in reservoirs

Daniel Montagud[1,*], Juan M. Soria[1], Xavier Soria-Perpiñà[2], Teresa Alfonso[1] and Eduardo Vicente[1]

[1] Cavanilles Institute of Biodiversity and Evolutionary Biology (ICBIBE). University of Valencia, 46980-Paterna, Spain.
[2] Image Processing Laboratory. University of Valencia, 46980-Paterna, Spain.

[*] Corresponding author: damonta@alumni.uv.es

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ABSTRACT

A comparative study of four indices based on zooplankton as trophic state indicators in reservoirs

This study aims to examine four recently conducted trophic state indices that are based on the density of zooplankton and designed for estimating the trophic state of inland waters. These indices include two with formulations based on quotients or ratios, the Rcla and the Rzoo-chla, which were proposed and validated in the European project ECOFRAME (Moss *et al.*, 2003), and two with formulations based on the incorporation of a statistical tool comprising canonical correspondences analysis (CCA), the Wetland Zooplankton Index proposed in 2002 by researchers from McMaster University of Ontario (Lougheed & Chow-Fraser, 2002) and the Zooplankton Reservoir Trophic Index, an index recently designed by the Ebro Basin Authority and on which this manuscript is the first article. These indices were studied and applied in 53 heterogeneous reservoirs of the Ebro Basin. In addition, all were subsequently validated by Carlson's Trophic State Index based on the amount of chlorophyll *a* (Carlson, 1977), with significant differences found between them.

Key words: bioindicators, trophic state, Ebro Basin, zooplankton, canonical correspondence analysis, zooplankton reservoir trophic state index

RESUMEN

Estudio comparativo de cuatro índices basados en el zooplancton como indicadores de estado trófico en embalses

El presente estudio pretende estudiar cuatro índices de estado trófico realizados recientemente, basados en la densidad del zooplancton y diseñados para la estimación del estado trófico de las aguas continentales. Estos índices fueron dos cuya formulación se basa en cocientes o ratios, el Rcla y el Rzoo-chla que se propusieron y validaron en el proyecto europeo ECOFRAME (Moss et al.*, 2003), y dos cuya formulación se basa en la incorporación de una análisis estadísticos de correspondencias canónicas (CCA), el* Wetland Zooplankton Index *propuesto en el año 2002 por investigadores de la Universidad McMaster de Ontario (Lougheed & Chow-Fraser, 2002) y el* Zooplankton Reservoir Trophic Index*, índice diseñado recientemente por la Confederación Hidrográfica del Ebro y del cual el presente manuscrito representa su primer artículo científico al respecto. Dichos índices fueron estudiados y aplicados en 53 embalses heterogéneos de la Confederación Hidrográfica del Ebro. Además, todos ellos fueron validados a posteriori mediante el* Trophic State Index *de Carlson basado en la cantidad de clorofila* a *(Carlson, 1977), observándose diferencias significativas entre ellos.*

Palabras clave: *bioindicadores, estado trófico, lagos de la cuenca del Ebro, zooplancton, análisis de correspondencias canónicas,* zooplankton reservoir trophic state index

INTRODUCTION

Numerous studies conducted since the mid-1980s demonstrate the relationship between zooplanktonic communities and the trophic states of inland water bodies (e.g., Bays *et al.*, 1983; Gulati, 1983; Blancher, 1984). This premise led to the study of zooplankton as a possible bioindicator of the ecological potential of different inland water bodies (e.g., Burns *et al.*, 1999; Caramujo & Boavida, 2000; Lougheed & Chow-Fraser, 2002).

Four indices based on zooplankton were studied in this paper, two of which do not present a statistical tool and two with a multivariate statistical component. The former indices are the ratio of big cladocers to total cladocers (Rcla) and the ratio of biomass zooplankton to the chlorophyll *a* concentration (Rzoo-chla), which are based on ratios or quotients. The latter are the Wetland Zooplankton Index (WZI) and the Zooplankton Reservoir Trophic Index (ZRTI), which include an analysis of canonical correlations (CCA) between environmental, physical and chemical variables and zooplankton taxa to observe the relationships between them and obtain coefficients or multiplication factors for each of the zooplankton species considered.

The Rcla and Rzoo-chla indices were proposed and validated in the European ECOFRAME project. They were studied in various shallow lakes in different European countries (Finland, Sweden, Estonia, Denmark, Germany, Poland, Ireland, the UK, the Netherlands and Spain) (Moss *et al.*, 2003).

The WZI index was proposed in 2002 by researchers at McMaster University in Ontario to determine the ecological potential of the Great Lakes in the northern United States and southern Canada (Lougheed & Chow-Fraser, 2002).

Finally, the ZRTI was proposed and produced by the Ebro River Basin Authority to determine the ecological potential of its reservoirs by considering zooplankton and other variables as quality indicators.

The ZRTI index has already been used in the Ebro Basin Authority's annual reports monitor-

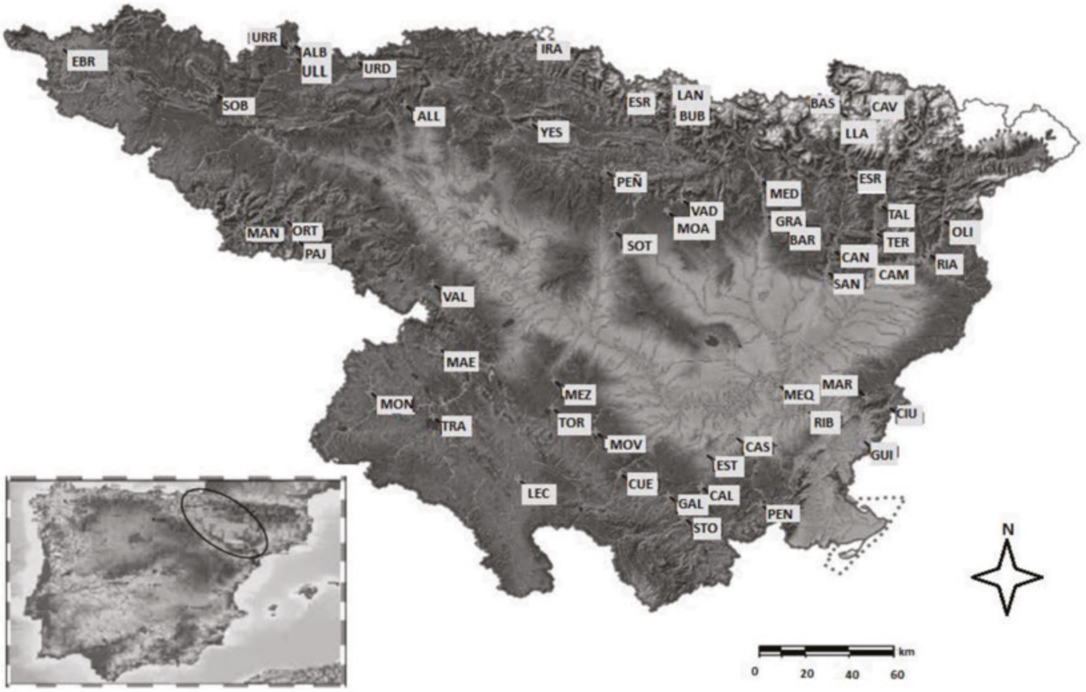

Figure 1. Location map of reservoirs in Ebro basin. *Mapa de la situación de los embalses de la Cuenca del Ebro.*

ing the water quality of its reservoirs (CHE, 2009), but no study has been conducted to review its suitability and operation for quality assesment, except for its initial presentation (Mellado *et al.*, 2010).

This study was motivated by the need to formulate and implement a biological index for determining the ecological potential of reservoirs, since no such index has yet been formulated specifically for reservoirs, and, therefore, the need for such an index is urgent for compliance with the European Water Framework Directive (EC 2000).

This study validates the new ZRTI index, as it demonstrates its correct functioning in the reservoirs studied, as well as in comparisons with other indices, the functionalities of which are already consolidated.

Therefore, this study aims to analyze various indices of diverse origin, all of them based on zooplankton, to observe differences among them and validate them by means of the Trophic State Index (Carlson's TSI), an index based on the concentration of chlorophyll *a* (Carlson, 1977) in several water samples. Which index is most efficient in diagnosing environmental quality? And is it possible to use indices produced in other regions in our area of study? This paper intends to investigate these questions.

METHODOLOGY

Data have been collected from samples of environmental variables and zooplankton density in 53 different reservoirs, all of which have heterogeneous characteristics and belong to the territorial demarcation of the Ebro Basin. Field sampling took place during the summer periods from 2014 to 2016 to determine the water quality of these reservoirs using the official methodology established for this purpose. These reservoirs present heterogeneous characteristics, since the Ebro River Basin has a wide territorial extension that covers a region that ranges from the Cantabrian high mountains to the Pyrenees and the Iberic Mountains to the Catalan coastal zone, presenting climatic and lithological differentiations that attributes different characteristics to each reservoir. The reservoirs, with their abbreviations, are geolocated in Figure 1. The abbreviations, the year of sampling and the various heterogeneous properties of each site can be found in Table S1 (see supplementary information at http://www.limnetica.net/en/limnetica).

In accordance with the official methodology (Vicente *et al.*, 2005), a single sampling station was established in the deepest part of each reservoir, at a distance of between 100 and 300 meters from the dam.

Physical and chemical variables

The depth of the photic zone was determined by measuring light penetration using a PAR quantometer. In addition, in situ water transparency was determined using the Secchi disc technique. The temperature, dissolved oxygen, conductivity and pH of the water were measured in situ continuously along the vertical profile approximately every 5 cm by means of the multiparametric probe Sea-Bird 19 plus V2 (SBE19). An integrated water sample was collected from the photic zone using the 25 mm diameter ballasted tube technique, or, in accordance with the official methodology, at least a 6 m deep column if the depth of the photic zone was lower than 6 m (Vicente *et al.*, 2005). The aliquots corresponding to the analyses were separated into different bottles.

The analytical methodology described in APHA 2540D (APHA, 1998) was used to determine total suspended solids and total volatile solids. The indophenol method was used in the determination of ammonium (Verdow *et al.*, 1978). Total nitrogen was obtained using the analytical methodology described in APHA method 4500-N C (APHA, 1998). For the determination of total phosphorus, the methodology described in APHA method 4500-P B/APHA 4500-P C (APHA, 1998) was used, with a limit of detection and quantification of 0.2 μg P/L, and the determination of chlorophyll *a* was obtained following the analytical methodology described in method APHA 1200 H (APHA, 1998). For the determination of dissolved carbon, the samples were manipulated and analyzed according to the protocols described in Soria (2017), using the non-purgeable organic carbon method in a Shimadzu TOC-VCSH/CSN analyzer.

Biological variables

The biological variable considered in this study was zooplankton density expressed in individuals/litre. The guides used for the taxonomic identification of zooplankton were Stella (1984), Segers (1995), Nogrady *et al.* (1995), Benzie (2002) and Nogrady & Segers (2002). The depth of collection of the zooplankton sample was determined by trying to incorporate the zone of the beginning of oxygen decline, which generally coincides with the end of the photic zone. This area is the richest in this type of fauna during the day (Miracle *et al.*, 1983). Using a Ruttner vertical hydrographic bottle with a capacity of 2.6 L, two samples of 2.6 L each were collected and filtered by means of 20 µm pore nytal mesh, which was preserved in formaldehyde at a final concentration of 4-5 %. For the capture of the qualitative zooplankton present in the medium, a vertical trawl was carried out in all reservoirs with a 50 µm pore zooplankton net, which was also preserved in formalin and used for taxonomic studies. For the determination of the zooplanktonic community a Sedgewick Rafter-type counting chamber was used and implemented using a stereoscopic inverted microscope to count the crustaceans, while an optical microscope was normally required for their identification, as well as for the counting-identification of rotifers, for which an inverted microscope was also used. A dye (Rose Bengal) was used to facilitate counting and identification when necessary.

Calculation of the indices

As indicated above, four indices were used, two based on ratios, the Rcla and the Rzoo-chla, and two based on the integration of zooplanktonic species in a statistical analysis of CCA, the WZI and the ZRTI. In the CCAs performed for the calculation of WZI and ZRTI, a Montercalo permutation test was also performed with N = 999.

Rcla and Rzoo-chla indices

The Rcla and Rzoo-chla indices were formulated in the ECOFRAME project for the study of the ecological quality of shallow lakes (Moss *et al.*, 2003).

The Rcla index is the result of the ratio between the number of big cladocers to the total number of cladocers (in this work, species of the genera *Diaphanosoma* and *Moina*, as well as the species *Leydigia leydigii*, *Holopedium gibberum* and *Simocephalus vetulus*, all of which are between 0.2 – 5.0 mm long, were considered big cladocers), and the Rzoo-chla index is the result of the ratio between the total zooplankton biomass and volume in mg/L. The biomass was estimated from biovolume calculations, assuming a specific gravity of 1 mm^3 (Wetzel *et al.*, 2000), and the phytoplankton were expressed as chlorophyll *a* concentration.

WZI index

Abbreviated from Wetland Zooplankton Index (Lougheed & Chow-Fraser, 2002). This index was developed in the USA for assessing the quality of aquatic ecosystems of different types (palustre, lake, river, etc.) using zooplankton communities. Eight physical and chemical variables are used in its CCA: temperature, dissolved oxygen, conductivity, pH, total suspended solids, chlorophyll *a*, phosphorus and total nitrogen.

The index uses tolerance (*Ti*) and optimal (*Ui*) values for different genera and species of zooplankton (rotifers, copepods and cladocers). These values have been obtained from the results of the first axis in the CCA according to the authors' methodology.

The mathematical expression of the index is:

$$\mathrm{WZI} = \frac{\sum_{i=1}^{n} Y_i T_i U_i}{\sum_{i=1}^{n} Y_i T_i}$$

Where *Yi* is the abundance (ind/L) of species *i*, *Ti* is its tolerance (values 1-3) and *Ui* is its optimum (values 1-5). The index therefore varies from 1 (poor indicator) to 5 (maximum quality indicator).

The optimum *U*, or center of distribution, and the tolerance *T*, or range of distribution, values were determined for each taxon based on a CCA using these collapsed taxonomic groupings. The location of a taxon on CCA axis 1 is represented by the center of distribution along the primary synthetic axis formed by several key environmental variables (Ter Braak *et al.*, 1995). In general, taxa tolerant of degraded conditions (U = 1–2) had broader tolerance (T = 1), while those less tolerant (U = 4–5) of degraded conditions often had a narrower tolerance (T = 3). The U and T values for each species obtained for each zooplanktonic species can be found in Table S2 (see supplementary information at http://www.limnetica.net/en/limnetica).

ZRTI index

As mentioned above, one of the novel aspects of this publication is the incorporation of the ZRTI, an index used to obtain the ecological potential of the reservoirs of the Ebro River Basin. As in the WZI, eight physical and chemical variables were used in the CCA: Secchi's disk values, photic zone depth, total volatile solids, total ammonium, total dissolved carbon, chlorophyll *a*, phosphorus and total nitrogen. For zooplankton, the density values of all taxa were identified.

For the calculation of the ZRTI, each taxon was assigned a tolerance eutrophy value *ti* ranging from 1 (minimum tolerance) to 15 (maximum tolerance), calculated from its relative position in the trophic state gradient as defined by the first axis of ordering and according to the procedure established here:

-The lowest weight obtained in the CCA was divided into 7.5 parts, and the result of the division is the threshold value for each category (from 1 to 7).

-The highest weight obtained in the CCA was divided into 7.5 parts, and the result of the division is the threshold value for each category (from 9 to 15). Central category around 0 value is the value 8.

The index is the weighted sum (with the relative density of each species *di* as a weighting factor) of the tolerance values of the species present in each reservoir:

$$\text{ZRTI} = \Sigma \text{ di ti}$$

Where *di* is the relative density and *ti* is the tolerance value of species *i*.

The ZRTI index ranges from 1 to 15, with ecological classes (called Ecological Potentials) being calculated by equitably dividing this range into five quality classes as shown in Table 1.

The ti values obtained for each zooplanktonic species can be found in Table S2.

Index validation

Carlson's TSI (Carlson, 1977) based on the concentration of chlorophyll *a*, an index with consolidated functionality, was used to validate the indices, and a conventional linear regression was subsequently carried out using the *Excel* software package.

Table 1. Ecological potential value classes for the different índices. *Porcentaje de representación. Clases de valores del potencial ecológico para los diferentes índices.*

Ecological potential classes	Optimal	Good	Moderate	Deficient	Bad
Rcla Range	>0.5	0.3-0.5	0.1-0.3	0.05-0.1	<0.05
Rzoo-chla Range	> 70	50-70	20-50	10-20	<10
WZI Range	>4.2	3.4-4.2	2.6-3.4	1.8-2.6	<1.8
ZRTI Range	<3.8	3.8-6.6	6.6-9.4	9.4-12.2	>12.2
Carlson's TSI Range	<20	20-40	40-60	60-80	>80

Statistical processing

The physical and chemical variables used to calculate the indices were temperature, dissolved oxygen, water conductivity, pH, total suspended solids, total nitrogen and phosphorus, chlorophyll *a*, water transparency, depth of the photic zone, total volatile solids, ammonium and total dissolved organic carbon. The multivariate statistical analysis of CCAs and the Montecarlo permutation test for the WZI and ZRTI indices was performed using the free downloadable software package PAST version 3.18 (Hammer *et al.*, 2001). All data used have been transformed to a logarithmic neperian scale for standardization, except pH.

RESULTS

Physical and chemical variables

The mean, maximum and minimum values of each physical and chemical variable studied are given in Table 2. In general, the values that correspond to indicators of higher quality are found in the reservoirs of the Pyrenean area (Cavallers, Llauset & Canelles), while the values corresponding to low-quality indicators are found in small, medium or low reservoirs, as is the case of Lechago, Terradets, or located in poor quality rivers like Oliana, Cueva Foradada and Moneva. Most reservoirs present values indicating intermediate qualities of the variables used.

With respect to zooplankton, 75 different taxa were identified across all the reservoirs. The taxonomic group with the most species found was that of rotifers with 48 different species, followed by the taxonomic group of cladocers with 16 different species, and the taxonomic group with the fewest species was that of the copepods with 11 different species.

If we look at the density of total zooplankton, the reservoir with the highest value was Moneva with 5911 individuals/L, while the reservoir with the lowest value of zooplankton density was Sopeira reservoir with 8 individuals/L.

But if we focus on the density of the large cladocerans (see Methodology section), a variable necessary for the calculation of the Rcla index, the highest value corresponds to the Rialb reservoir with 55 individuals/L, while the reservoir with the lowest density of large cladocerans is Llauset, in which no individual was found.

Table 2. Mean, maximum and minimum of physical and chemical variables. *Promedio, máximo y mínimo de las variables fisicoquímicas.*

Variables	Average	Maximum	Minimum
Temperature (ºC)	**18.61**	**27.50**	**10.50**
Oxygen (mg/L)	**8.21**	**10.10**	**4.75**
Conductivity (µS/cm)	**371.96**	**1372.00**	**12.29**
pH	**8.12**	**8.64**	**6.98**
Solids (mg/L)	**3.27**	**16.79**	**0.07**
N total (µM)	**51.74**	**306.78**	**8.48**
P total (µM)	**0.36**	**2.61**	**0.03**
Chlorophyll *a* (µg/L)	**3.24**	**21.27**	**0.54**
Secchi disk depth (m)	**4.18**	**17.20**	**0.75**
Photic zone depth (m)	**11.84**	**43.00**	**3.10**
Volatile solids (mg/L)	**0.97**	**4.60**	**0.05**
Ammonium (µM)	**1.47**	**6.83**	**0.09**
Dissolved carbon (mg/L)	**1.74**	**3.57**	**0.37**

Analysis of Canonical Correspondences

In the CCA with the same physical and chemical variables corresponding to the WZI index, 31.66 % of the cumulative variance on axis 1 (the only axis of the analysis of interest to us) was obtained with a p value of 0.001 in the Monte Carlo permutation test. It was observed that those variables associated with eutrophication and poor water quality (temperature, conductivity, total solids, chlorophyll *a*, nitrogen and total phosphorus) had a negative weight on the first axis, while dissolved oxygen, associated with higher water quality (Bengraïne & Marhaba, 2003), had a positive weight. The pH variable was at the midpoint, value 0. The variable with the most negative weight was conductivity, while dissolved oxygen had the most positive weight on the first axis.

In this same CCA, it was observed that those species belonging to the Cladocera taxonomic group had positive values, while the species belonging to the Rotifera taxonomic group were located more to the negative side of the axis. The species with the most positive statistical weight

Table 3. Result of the indices in the reservoirs. Grey scale for quality as in Table 1. *Resultado de los índices en los embalses. Escala de grises para la calidad como en la tabla 1.*

Reservoir	Rcla Value	Rzoo-Chl*a* Value	WZI Value	ZRTI Value	Carlson's TSI
Albiña	1.00	97.37	3	7	43
Alloz	0.93	55.60	3	8	41
Barasona	0.00	11.44	3	7	45
Baserca	1.00	28.88	3	6	41
Búbal	0.96	66.56	3	7	39
Calanda	0.98	80.08	3	9	37
Camarasa	0.10	59.99	3	8	42
Canelles	0.84	30.79	3	7	44
Caspe	0.93	71.64	3	10	40
Cavallers	1.00	118.29	3	5	37
Ciurana	0.51	44.19	3	8	38
Cueva Foradada	1.00	108.19	3	9	44
Ebro	1.00	27.53	3	8	42
Escales	0.91	30.95	3	7	43
Escarra	0.90	64.88	3	6	38
Estanca	1.00	32.33	3	9	45
Gallipuen	0.09	401.62	3	9	40
Grado	0.00	17.71	3	8	36
Guiamets	0.23	12.07	3	8	48
Irabia	0.70	19.93	3	8	44
Lanuza	0.99	142.66	3	7	37
Lechago	1.00	81.40	4	11	45
Llauset	*No cladocers found*	1.13	3	5	35
Maidevera	0.11	76.64	3	8	51
Mansilla	0.84	9.67	3	7	43
Margalef	1.00	30.22	3	8	46
Mediano	0.19	35.38	3	7	41
Mequinenza	0.45	92.86	4	9	46
Mezalocha	0.33	24.10	3	8	48
Montearagón	0.19	11.50	3	7	43
Monteaguda de las Vicarias	0.98	71.02	4	11	44
Moneva	0.94	77.34	4	11	47
Oliana	0.65	26.22	3	8	51
Ortigosa	1.00	9.87	3	8	39
Pajares	0.99	27.56	3	8	42
Pena	0.98	85.55	3	7	36
La Peña	0.00	7.56	3	8	47
Rialb	0.59	98.02	3	8	45
Ribarroja	0.48	57.18	3	9	46
Santa Ana	0.52	12.82	3	7	41
Sobron	0.62	17.92	3	9	55
La Sotonera	0.05	19.77	3	9	45
Santolea	0.97	46.42	3	8	38
Talarn	1.00	17.41	3	7	45
Terradets	1.00	1.56	3	7	41
Las Torcas	0.58	39.20	3	8	43
La Tranquera	0.10	58.66	3	9	50
Ullivarri	0.04	124.17	3	9	42
Urdalur	0.77	12.06	3	8	40
Urrúnaga	0.04	6.63	3	7	49
Vadiello	0.34	12.25	3	7	39
El Val	0.64	15.07	3	8	61
Yesa	0.80	5.25	3	7	42

on the first axis was *Holopedium gibberum*, while the species with the most negative value was *Hexarthra oxyuris*.

In the CCA with the physical and chemical variables established by the ZRTI methodology, 32.0 % of the cumulative variance on axis 1 was obtained with a *p* value of 0.001 in the Monte Carlo permutation test. It was observed that, contrary to the previous case, the physical and chemical variables associated with a higher water quality were located in the negative part of the axis (transparency and depth of the photic zone), while the variables associated with poor quality were located at the most positive part of the axis (total volatile solids, ammonium). The variable with the most negative value in the first axis was the depth of the photic zone, and the total carbon dissolved was the variable with the most positive value on the first axis.

Regarding zooplankton, in the CCA of the ZRTI, it was observed that the species belonging to the Cladocera taxonomic group had more negative values, while the species belonging to the Rotifera taxonomic group were located more to the positive part of the axis. The species with the most negative statistical weight on the first axis was *Holopedium gibberum*, while the species with the most positive value was *Hexarthra oxyuris*, the same findings as those of the statistical analysis for WZI, which used different environmental variables.

Index results

The results obtained in the application of the various indices in all 53 reservoirs analyzed can be seen in Table 3.

According to the Rcla index, 67 % of the total reservoirs are ultraoligotrophic, 8 % oligotrophic, 12 % mesostrophic, 4 % eutrophic and 10 % hypertrophic. Without focusing on the Rzoo-chla index, 28 % are ultraoligotrophic, 11 % are oligotrophic, 30 % are mesotrophic, 21 % eutrophic and 9 % hypertrophic.

The WZI, ZRTI and Carlson's TSI indices do not establish any reservoir as either ultraoligotrophic or hypertrophic. More specifically, according to WZI, 8 % of the reservoirs are oligotrophic and 92 % are mesostrophic. The ZRTI also states that 8 % of the total number of reservoirs are oligotrophic but that 85 % are mesostrophic and 8 % eutrophic. Finally, the Carlson's TSI index states that 23 % of reservoirs are oligotrophic, while 68 % are mesostrophic and 9 % eutrophic.

Validations

As stated in the previous section, all indices were related and compared with Carlson's TSI. The statistical values obtained in the various regressions can be seen in Table 4.

The least significant values were those obtained in the two indices based on quotients or ratios, the Rcla and the Rzoo-chla, with the Rzoo-chla being more insignificant. Among the indices based on CCA statistics, ZRTI was the most significant.

In addition, for greater reliability of the conclusions, an additional validation of the ZRTI was carried out using only data from 2017, as these data are the most recently sampled. The critical value in Pearson's r for this validation turned out to be less than 0.001 (Dunnett, 1964), as shown in Table 4.

DISCUSSION

This study demonstrated the relationship between the trophic status of water bodies and the zooplankton community present, indicating that these organisms are suitable for the development of biological indices for the determination of ecological potential, as stated by several authors (eg., Ochocka *et al.*, 2016; Gilbert *et al.*, 2017).

Table 4. Result of index validations with Carlson's TSI. N = 53 for 2014-16 data; N = 40 for 2017 data. *Resultado de las validaciones de los índices con el TSI de Carlson. N = 53 en datos del 2014-2016; N = 40 en datos del 2017.*

Index	R Coeficient values	R Pearson critical values
RCLA	0.277	0.025
Rzoo-Chl*a*	0.180	>0.05 NO SIGNIFICANT
WZI	0.296	0.025
ZRTI	0.332	0.01
ZRTI 2017	0.504	<0.001

This study also found differences between the various indices studied. We observe many discrepancies in the allocations of trophic states depending on which indices are considered. While the results obtained in the statistical indices are related to the trophic state established by Carlson's TSI, a reference index, the indices based on quotients or ratios show no relation to the result obtained from Carlson's TSI. For example, the Yesa reservoir is classified as a mesotrophic reservoir by the WZI and ZRTI statistical indices and Carlson's TSI, while the Rcla index classifies it as ultraoligotrophic and the Rzoo-chla as hypertrophic.

It is observed that the ecological classification of reservoirs according to the indices based on CCA statistics is related to the results obtained in Carlson's TSI, while the classification of reservoirs according to the results obtained in the application of the indices based on quotients or ratios, Rcla and Rzoo-chla, have no apparent relationship either between them or with the other indices, including Carlson's TSI.

As for the validation of the different indices with respect to Carlson's TSI, we observed that the indices presenting statistics in their mathematical algorithm are statistically more correlated than the indices based on ratios, among which the Rzoo-chla was found to be not significant.

Although a more specific study of why these discrepancies exist and a reformulation of the indices based on quotients or ratios to enhance performance in this type of aquatic mass is necessary, one possible reason for their poor performance is that they were designed in the ECOFRAME project (Moss *et al.*, 2003) for shallow lagoons in several European countries; therefore, applying them to highly modified waters (reservoirs) in various regions gave an unsatisfactory result. Regardless, as already mentioned, among the indices that were studied in the present work, the one that adjusted worst to the trophic state of the reservoir was the Rzoo-chla, an index based on the ratio between the total existing zooplankton and the phytoplanktonic biomass expressed by the amount of chlorophyll *a*. One reason for its functional weakness could lie precisely in its formulation, since it assumes the same properties of all zooplankton species, even though many studies disprove this, and it could result in co-cataloguing species of zooplankton bioindicators of higher water quality that in most cases require favorable characteristics for their development with zooplankton species that have the widest trophic spectrum (Gannon *et al.*, 1978).

The statistical part of this study reflected the existence of zooplankton species indicating good ecological potential. For the CCAs carried out for the calculations of both WZI and ZRTI, the species belonging to the Cladocera taxonomic group were associated with a good ecological quality, namely the species *Cyclops abyssorum* and *Holopedium gibberum*, the latter being the species associated with better conditions of ecological quality in both cases.

Conversely, in both cases, species belonging to the taxonomic group of the Rotifera were in the parts of the axis related to bad ecological status; specifically, the species *Hexarthra oxyuris*, *Hexathra mira*, *Keratella tropica* and *Keratella quadrata* indicated low ecological quality.

These conclusions are consistent with most studies related to zooplankton as an ecological indicator (e.g., Pinto-Coelho *et al.*, 2005; Ejsmont-Karabin, 2012; Haberman *et al.*, 2014; Azevedo *et al.*, 2015), which find more individuals of the Rotifera taxonomic group in low quality areas and of the Cladocera taxonomic group in areas of higher ecological quality. These conclusions also agree with several studies of the populations of zooplankton in eutrophic lagoons in Spain, including *Lavajo de Abajo de Sinarcas* (Sahuquillo & Miracle, 2010) and *La Albufera* (Vicente & Miracle, 1992), that found that the most abundant species are of the taxonomic group of the Rotifera, specifically the species *Hexathra mira*, *Keratella quadrata* and *Keratella tropica*. Along the same lines, several studies carried out in reservoirs of the Júcar river detected the presence of individuals from the Rotifera taxonomic group in reservoirs of low water quality (García-Chicote *et al.*, 2017).

In the same way, this study was able to validate the correct functionality of the new trophic status index based on the ZRTI. This index has given the best statistical results among those studied here, with an r-Pearson *p*-value (Dunnett, 1964) lower than 0.001 in its validation with Carlson's TSI using 2017 data (Table 4). This shows the suitability of this index for this type of aquatic reservoir, so that, based on the results previously mentioned that have been evidenced in this work, it is possible to validate the regulatory use of this index for obtaining the trophic status of reservoirs in the various reports monitoring water quality that the Ebro Basin Authority has been consistently conducting. It would also be of interest to validate the methodology and results in other river basins, as well as to apply it to other lagoon ecosystems.

Finally, a linear correlation has been made between the values obtained in the application of the WZI and ZRTI indices in all the reservoirs studied, showing a significant correlation between the two indices with an r coefficient of 0.865 and Pearson's r critical value below 0.001 (Dunnett, 1964). Given these results and that the WZI index was designed for lakes and the ZRTI index for reservoirs, the ZRTI should be studied for possibilities of application in lakes and beyond to expand its use.

In addition, based on the results of this study, it is recommended that the Rcla and Rzoo-chla indices be revised to ensure that they function correctly in determining ecological potential outside the regions for which they were designed and that the WZI index be corrected to accurately determine the ecological potential of the reservoirs of the Ebro River Basin, though it is recommended that the new ZRTI index be used for this purpose because it is the most efficient index in this context.

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Development of a zooplankton biotic index for trophic state prediction in tropical reservoirs

Bruno Paes De-Carli[1,2,*], Adriano Bressane[3], Regina Márcia Longo[3], Agatha Manzi-Decarli[2], Viviane Moschini-Carlos[1] and Marcelo Luiz Martins Pompêo[4]

[1] São Paulo State University (UNESP), Institute of Science and Technology, Environmental Sciences Graduate Program, Campus at Sorocaba city, 03 March Avenue, 511, Brazil.
[2] Paulista University (UNIP), Institute of Health, Campus at Santos city, Francisco Manoel Avenue, unnumbered, Brazil.
[3] Pontifical Catholic University of Campinas (PUCCAMP), Faculty of Environmental engineering, Dom Pedro I Highway (SP-065), Brazil.
[4] Department of Ecology (IB-USP), University of São Paulo, São Paulo, Brazil.

* Corresponding author: bpdecarli@yahoo.com.br

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ABSTRACT

Development of a zooplankton biotic index for trophic state prediction in tropical reservoirs

Reservoirs are built mainly for public supply and power generation. However, water quality is almost always compromised by discharge of domestic and industrial sewage, as well as by agricultural residues. Several ecological indices are currently used to analyze different impacts in this environment. The aim of this study was to develop a zooplankton index for tropical reservoirs. Limnological data were obtained from seven Brazilian reservoirs (Atibainha, Broa, Barra Bonita, Salto Grande, Rio Grande, Itupararanga, and Igaratá). Weighted values of ecological optimum were obtained through species response analysis (unimodal distribution) related to chlorophyll a concentration. The results obtained using the zooplankton index (ZBI) proposed had significant correlations with eutrophication indicators. Overall, poor and regular water quality were verified in most reservoirs, partially corroborating the zooplankton community index proposed for Brazilian reservoirs. For further progress, the approach presented here must be tested in other regions. In addition, the ecological indices derived from different aquatic communities should be integrated into a composite index.

Key words: zooplanktonic index, eutrophication, ecological potential, São Paulo State

RESUMO

Desenvolvimento de um índice biótico do zooplâncton para predição do estado trófico em reservatórios tropicais

Reservatórios são construídos principalmente para abastecimento público e geração de energia. No entanto, a qualidade da água é quase sempre comprometida pela descarga de esgoto doméstico e industrial além de resíduos agrícolas. Atualmente vários índices ecológicos são usados para diagnosticar impactos nesses ambientes. Este estudo teve como objetivo desenvolver um índice do zooplâncton para reservatórios tropicais. Dados limnológicos foram obtidos em sete reservatórios brasileiros (Atibainha, Broa, Barra Bonita, Salto Grande, Itupararanga e Igaratá). Os valores ponderados de ótimo ecológico foram obtidos através da análise de resposta das espécies (distribuição unimodal) relacionado com as concentrações de clorofila a. O índice do zooplâncton (ZBI) proposto apresentou correlação significativa com os indicadores de eutrofização. No geral, os reservatórios analisados apresentaram qualidade da água ruim e regular o que corrobora parcialmente com o índice da comunidade zooplanctônica de reservatórios brasileiros. Para futuros avanços, a abordagem proposta deve ser testada em outras regiões. Além disso, índices ecológicos derivados de diferentes comunidades aquáticas devem ser consolidados em um índice composto.

Palavras chave: *índice zooplanctônico, eutrofização, potencial ecológico, Estado de São Paulo*

INTRODUCTION

Reservoirs are used for different purposes, such as hydropower generation and storage of water for human consumption. At the same time, they are subject to different types of pressures that can cause loss of chemical and biological quality, thereby diminishing their ecological, economic, and cultural benefits (Breunig *et al.*, 2017; Cardoso-Silva *et al.*, 2018). Their monitoring, management, and protection requirements are essential to guarantee the best possible use (Lopez-Doval *et al.*, 2017). According to the Brazilian Environment Council and the Water Framework Directive (WFD), aquatic organisms and communities can be used as biological indicators to assess the quality of the environment. However, in Brazil, determination of classes to be used is still based on chemical analysis.

Bioindicators are living organisms such as plants, plankton, animals, and microbes that are used to assess ecosystem health. Each organic entity inside a biological system provides an indication of the health of its surroundings, such as plankton, which respond rapidly to changes in the surrounding environment and serve as an important biomarker and indicator of water pollution (Parmar *et al.*, 2016). Freshwater zooplankton communities consist mainly of protozoans, rotifers, and microcrustaceans (Pereira *et al.*, 2011; Cavan *et al.*, 2017; De-Carli *et al.*, 2018). Zooplankton play an important role in the pelagic food web as a mediator of nutrient and energy fluxes (Wetzel, 1995). Understanding factors that determine zooplankton abundance, composition, and dispersal provides information needed to improve plankton dynamic predictions and enhance effective water resource management and biodiversity conservation (Zhao *et al.*, 2017). Pollution-sensitive species are generally eliminated while more resistant species show high population growth rates (Matsumura-Tundisi & Tundisi, 2003). Furthermore, zooplankton may be considered a good indicator of environmental changes caused by impactful activities (Ferdous & Muktadir, 2009; Costa *et al.*, 2016; Hemraj *et al.*, 2017).

Biotic indices to monitor water quality are helpful tools for evaluating the health of rivers and lakes. In Brazil, water samples are analyzed

Table 1. Morphometric characteristics of the Salto Grande (SG), Barra Bonita (BB), Broa (BR), Itupararanga (IT), Atibainha (AT), Rio Grande (RG), and Igaratá (IG) reservoirs. Legend: maximum depth (Mdepth); sampling depth (Sdepth). *Características morfométricas dos reservatórios Salto Grande (SG), Barra Bonita (BB), Broa (BR), Itupararanga (IT), Atibainha (AT), Rio Grande (RG) e Igaratá (IG). Legenda: profundidade máxima (Mdepth); profundidade de amostragem (Sdepth).*

Sites	UTM coordinates x	y	Mdepth m	Sdepth m
SG-R	271746	7484625	5.5	4
SG-C	267855	7486087	8.4	3
SG-D	265792	7487792	11.0	3
BB-R	772898	7497007	16.4	4
BB-C	767108	7502593	18.3	7
BB-D	755107	7506492	22.6	8
BR-R	203139	7540974	3.2	2
BR-C	201694	7543600	6.4	2
BR-D	200605	7545526	13.0	2
IT-R	336225	7419553	8.2	2
IT-C	329679	7418759	10.8	6
IT-D	330027	7417799	14.8	5
AT-R	318857	7370288	8.9	1
AT-C	324042	7372330	19.1	1
AT-C5	324589	7378563	17.0	1
RG-R	267375	7388420	4.4	3
RG-C	262543	7385709	7.5	5
RG-D	256208	7386044	11.7	5
IG-R	365396	7439484	28.6	5
IG-2	360537	7437729	44.0	7
IG-B	359533	7432897	24.5	7

using mainly physical and chemical attributes because most biotic indices were developed in other countries and their effective application to Brazilian ecosystems requires significant research. In this sense, the zooplankton indices have been overlooked (EC 2000; Søndergaard *et al.*, 2005; Jeppesen *et al.*, 2011), unlike other biological communities such as macrophytes, phytoplankton, benthic invertebrates, and fish.

The relationship between zooplankton and water quality has been the subject of several studies (Gannon & Stemberger, 1978; Matsumura-Tundisi *et al.*, 2002; Sousa *et al.*, 2008; Sakamoto *et al.*, 2018). A wetland zooplankton index for water quality assessment was developed In North America (Lougheed & Chow-Fraser, 2002), using weighted values to formulate equations. Similarly, Hering *et al.* (2006) and Kane *et al.* (2009) developed a biotic integrity planktonic index and Carpenter *et al.* (2006) prepared a zooplankton index of biotic integrity in an estuarine environment. Ejsmont-Karabin (2012) compiled rotifer data from temperate lakes and pointed metrics for trophic state assessment. In Brazil, the Environmental Agency of São Paulo State, Brazil (CETESB, 2006), has applied biological indices since 2003, an important advance for environmental management of reservoirs, rivers, and streams. Here, we developed and validated predictive index for water quality using zooplanktonic communities of the tropical reservoirs.

MATERIAL AND METHODS

Study area

The Atibainha Reservoir is part of the Cantareira System that supplies water to the metropolitan region of São Paulo (Andrade *et al.*, 2015). The Salto Grande Reservoir is situated in the hydrographic basin of the Atibaia River (one of the tributaries of the Piracicaba River, upstream of the upper basin of the Paraná River) (Zanata & Espindola, 2002). The Barra Bonita Reservoir is situated in the Tietê River basin and also receives water from the Piracicaba River near the cities of Barra Bonita and Iguaraçu do Tietê (SP) (Ometo *et al.*, 2000; Petesse *et al.*, 2007). The Broa Reservoir is a part of the Tietê/Jacaré basin, located in the central region of São Paulo State, and encompasses three main rivers: Tietê, Jacaré-Guaçu, and Jacaré Pepira (Tundisi *et al.*, 2008). The Itupararanga Reservoir is situated in the Alto Sorocaba basin, one of the six sub-basins of Middle Tietê (Conceição *et al.*, 2015). Rio Grande is an arm partially isolated from the Billings Reservoir, covering the municipalities of São Paulo, Santo André, São Bernardo do Campo, Diadema, Ribeirão Pires, and Rio Grande da Serra (Moschini-Carlos *et al.*, 2010) (Table 1).

Sampling and procedures

Between June and October 2015, samplings were performed in seven reservoirs (Rio Grande arm, Barra Bonita, Broa, Salto Grande, Atibainha, Itupararanga, and Igaratá) totaling 21 points. One sample per zone at each reservoir was collected considering dam, central region, water inlet, and channels (Kimmel *et al.*, 1990) (Fig. 1).

Dissolved oxygen, pH, temperature, and electrical conductivity were determined in situ using a multiparameter probe (YSI mod 556 MPS). Transparency was measured using a Secchi disk (SD). To measure inorganic dissolved nitrogen (DIN), total phosphorus (TP), suspended solids and chlorophyll *a* (CLa), water-integrated samples were collected using a hose according to the photic zone (Table 2). The hose was introduced repeatedly until it reached the appropriate volume to perform the analyzes (Becker *et al.*, 2010). Trophic state index (TSI) was calculated based on the basis of TP and CLa indices according to Carlson (1977) adapted from Lamparelli (2004) using the following equations: $TSI (TP) = 10*(6- (1{,}77-0{,}42*(\ln PT)/\ln 2))$; $TSI (CLa) = 10*(6-((0{,}92-0{,}34*(\ln CLa))/\ln 2))$; $TSI = [TSI (TP) + TSI (CLa)] / 2$. Sampling sites are classified into ultraoligotrophic ($TSI < 47$), oligotrophic ($47 < IET < 52$), mesotrophic ($52 < IET \leq 59$), eutrophic ($59 < IET < 63$), supereutrophic ($63 < IET < 67$), hypertrophic $IET > 67$.

Zooplankton samples were collected at the respective water collection sites using a plankton net (68 µm) at the limit of the photic zone (SD*2.7, Cole, 1979). Filtered volume was estimated by measuring depth trawl and radius of the net mouth. Taxonomic identification was based on Koste (1978), Elmoor-Loureiro (1997), Nogrady & Segers (2002), Silva (2008) and Perbiche-Neves *et al.* (2015). A Sedgwick-Rafter chamber with 1 mL capacity was used for density estimative (ind./m^3), with aliquots from the total homogenized sample.

Zooplankton Biotic Index

We developed a novel Zooplankton Biotic Index (ZBI) that uses data from species unimodal response analysis (Leps & Smilauer, 2003) using the response curves form a statistical model of

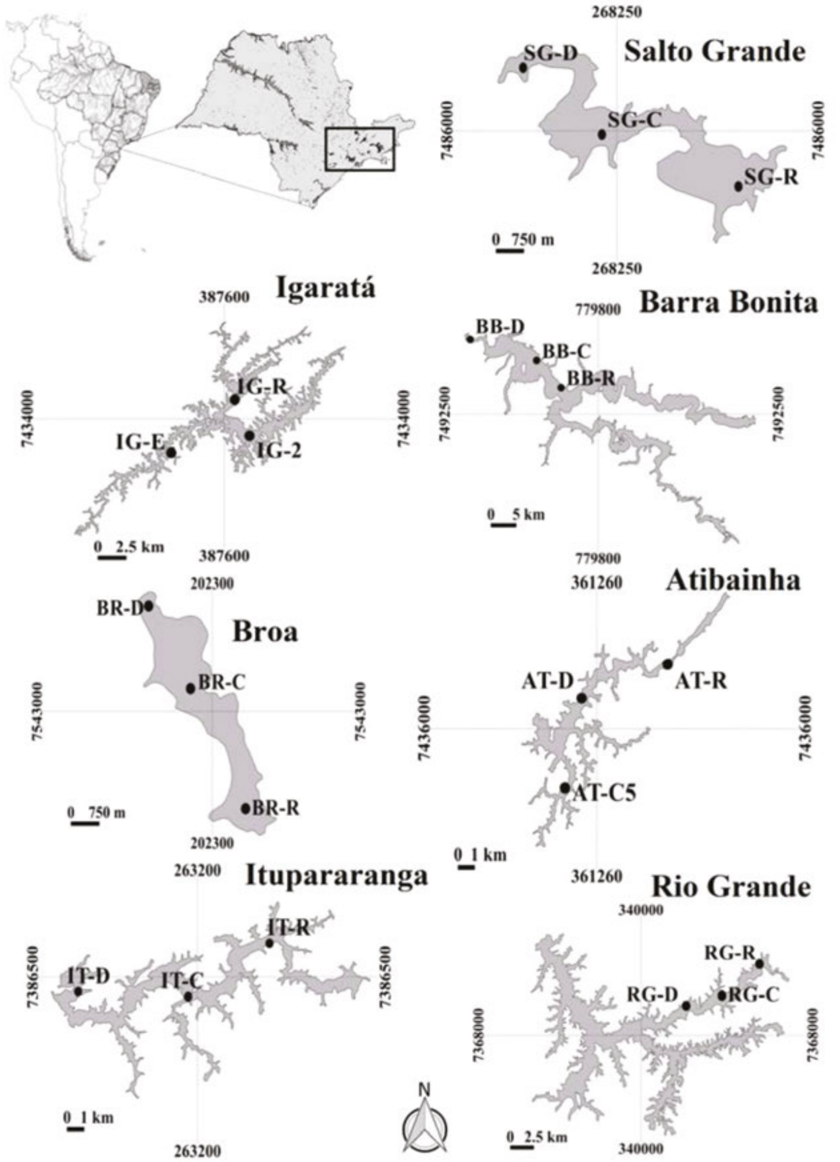

Figure 1. Map showing 21 sampling sites from studied reservoirs. Legends: Salto Grande (SG), Barra Bonita (BB), Broa (BR), Itupararanga (IT), Atibainha (AT), Rio Grande (RG) and Igaratá (IG). River inlet (R), central (C, 2), dam zones (D), and water outlet (E). *Mapa demonstrando os 21 pontos de amostragem dos reservatórios analisados. Salto Grande (SG), Barra Bonita (BB), Broa (BR), Itupararanga (IT), Atibainha (AT), Rio Grande (RG) e Igaratá (IG). Entrada de água (R), centro (C,2), zona de barragem (D) e saída de água (E).*

zooplankton composition in relation to CLa (Jamil *et al.*, 2014). From the model, it is calculated the probability that a particular species will be observed at a given chlorophyll *a* value. By determining this probability for a range of chlorophyll *a* value, we can select the value that gives the highest probability of observing the specific taxon. The CLa value is the maximum likelihood estimate (ter Braak & van Dame, 1989). Thus, optimum values were calculated through a Gaussian model along with CLa concentration to make up the final index as follows:

$$\mathrm{OP(sp)} = \frac{\sum_{i=1}^{n} \mathrm{Env}_i \text{ x } \mathrm{Abund}_i}{\sum_{i=1}^{n} \mathrm{Abund}_i}, \qquad (1)$$

OP (sp) corresponds to the optimum value of each species, Envi represents the value of the environmental variable (CLa) in the ith sample, and Abundi corresponds to the abundance of the zooplankton species in the ith sample.

From this, we adapted the equation shown below, which aims to predict the trophic state:

$$\mathrm{ZBI} = \frac{\sum_{i=1}^{n} \mathrm{OP}_{sp} \text{ x } \mathrm{Abund}_i}{\sum_{i=1}^{n} \mathrm{Abund}_i}, \qquad (2)$$

The following classification was adopted for ZBI: < 7.22, good; 7.23 to 11.53, regular; and 11.54 to 15.84, poor. This scale was developed using the Sturges formula for determining classes and is based on trophic state index correlation (Oliveira *et al.*, 2008).

Zooplanktonic Community Index for reservoirs (ICZ) was calculated using a water quality diagram, which links the Calanoida/Cyclopoida ratio and CLa concentrations (CETESB, 2006). This index does not present absolute values, but rather water quality classes (very poor, poor, regular, and good).

Validation

The ZBI index has been tested applying the present survey data observing the association between index and limnological variables. Spearman coefficient (r_S) was used to analyze the degree of correlation between variables. A regression analysis was used for verification as the calculated indices vary according to the environmental variables. For this, we used the first principal component axes (obtained from phosphorus and chlorophyll *a*), trophic state index, total phosphorus, chlorophyll *a* and correlated with proposed index. In order to achieve this analysis assumptions data were transformed by the ranging method (Milligan & Cooper, 1988), where the observed value less minimum is divided by maximum less minimum. All statistical analyses were carried out using PAST version 3.17 (Hammer *et al.*, 2001).

RESULTS

The Igaratá Reservoir has the deepest sites and has the highest levels of water transparency. High electrical conductivity and nutrient enrichment (TP and DIN) were observed in Salto Grande and

Table 2. Limnological parameters and methods used in Salto Grande (SG), Barra Bonita (BB), Broa (BR), Itupararanga (IT), Atibainha (AT), Rio Grande (RG), and Igaratá (IG) reservoirs. *Parâmetros limnológicos e métodos utilizados nos reservatórios Salto Grande (SG), Barra Bonita (BB), Broa (BR), Itupararanga (IT), Atibainha (AT), Rio Grande (RG) e Igaratá (IG).*

Variables	Method	References
Suspended solids (mg/L)	Gravimetry	Teixeira *et al*. (1965)
Total phosphorus (μg/L)	spectrophotometric	Valderrama (1981)
Nitrate (μg/L)	spectrophotometric	Mackereth *et al*. (1978)
Nitrite (μg/L)	spectrophotometric	Mackereth *et al*. (1978)
Ammonium (μg/L)	spectrophotometric	Koroleff (1976)
Inorganic dissolved nitrogen (μg/L)	$\sum NO_2- + NO_3- + NH_4+$	Wetzel & Likens (1991)
Chlorophyll a (μg/L)	spectrophotometric	Lorenzen (1967)

Table 3. Limnological variables obtained from water integrated samples in São Paulo state reservoirs. Legends: Salto Grande (SG), Barra Bonita (BB), Broa (BR), Itupararanga (IT), Atibainha (AT), Rio Grande (RG), and Igaratá (IG). River inlet (R), central (C, 2), dam zones (D), and water outlet (E). Temperature (T), dissolved oxygen (DO), electrical conductivity (EC), Secchi disk (DS), suspend solids (SS), inorganic dissolved nitrogen (DIN), total phosphorus (TP), chlorophyll *a* (CLa). Underlined data indicates minimum and bold maximum values. (*) Value corresponding to half detection limit (13 μg/L). *Variáveis limnológicas obtidas de amostras integradas de água nos reservatórios do Estado de São Paulo. Legendas: Salto Grande (SG), Barra Bonita (BB), Broa (BR), Itupararanga (IT), Atibainha (AT), Rio Grande (RG) e Igaratá (IG). Entrada de água (R), centro (C,2), zona de barrage (D) e saída de água. Temperatura (T), oxigênio dissolvido (DO), condutividade elétrica (EC), disco de Secchi (DS), sólidos em suspensão (SS), nitrogênio inorgânico dissolvido (DIN), fósforo total (TP), clorofila* a *(CLa). Dados sublinhados indicam mínimos e valores em negrito máximo. (*) Valor correspondente a metade do limite de detecção (13 μg/L).*

Sites	T °C	DO mg/L	pH	EC μS/cm	DS m	SS mg/L	DIN μg/L	TP μg/L	CLa μg/L
SG-R	20.87	3.22	6.78	**410**	1.24	8.00	908.32	**115.30**	26.73
SG-C	20.92	3.16	7.12	363	1.07	8.14	355.78	100.19	13.75
SG-D	21.25	2.71	6.86	340	1.19	7.71	399.25	69.12	19.38
BB-R	22.23	4.01	7.01	274	1.55	3.90	**2063.54**	95.15	15.64
BB-C	20.99	3.56	6.80	258	2.50	3.10	845.29	64.93	11.63
BB-D	22.41	2.80	6.88	247	2.90	3.00	382.94	70.80	3.74
BR-R	22.57	5.69	7.11	18	0.63	7.75	66.90	28.83	30.07
BR-C	22.57	4.26	7.46	17	0.62	6.75	27.75	18.75	29.74
BR-D	21.56	4.11	7.90	17	0.62	**10.33**	38.01	27.99	**38.09**
IT-R	17.91	4.40	7.28	83	1.23	6.40	89.25	28.83	8.82
IT-C	19.38	2.97	6.59	81	1.61	2.80	60.66	13.71	5.35
IT-D	19.25	3.65	6.81	80	1.39	3.60	63.67	14.55	9.76
AT-R	**24.96**	4.08	7.51	41	1.88	2.57	244.32	7.50*	7.64
AT-C	24.33	3.52	7.85	40	2.65	2.12	102.67	7.50*	5.51
AT-C5	24.17	2.57	**7.98**	39	2.38	2.25	73.74	7.50*	2.17
RG-R	22.59	2.61	2.61	108	1.12	4.17	571.39	33.86	5.16
RG-C	22.57	3.20	3.20	104	1.52	0.50	604.83	21.27	5.12
RG-D	22.61	**5.15**	5.15	105	1.71	6.83	663.52	7.50*	9.80
IG-R	22.57	4.94	5.76	33	**5.15**	0.60	106.40	7.50*	0.40
IG-2	22.66	3.51	3.74	35	3.95	0.90	104.73	17.07	0.67
IG-E	23.44	3.18	6.80	36	2.92	2.00	73.50	7.50*	3.61

Barra Bonita reservoirs. High chlorophyll *a* concentration was recorded at the Broa Reservoir. Based on the average CLa and TP indices, most sampling stations were classified as eutrophic, except for the Atibainha and Igaratá reservoirs, which were considered mesotrophic (Table 3).

Sixty-seven zooplankton taxa were identified, divided into Phylum Crustacea (Calanoida, Cyclopoida, Cladocera), aquatic insects belonging to the order Diptera, Phylum Rotifera, Phylum Protozoa, and Phylum Nematoda (Table 4). Phylum Rotifera was considered as having the greatest richness (33 taxa) and the Brachionidae family was considered most representative (14 taxa). Nauplius of Calanoida, nauplius and copepodites of Cyclopoida, *Thermocyclops decipiens* (Kiefer, 1929), *Thermocyclops minutus* (Lowndes, 1934), *Bosmina* sp., *Asplanchna* sp. (Eckstein, 1883), *Collotheca* sp. (Harring, 1913), *Kellicottia bostoniensis* (Rousselet, 1908), *Keratella cochlearis* (Gosse, 1851), and *Difflugia* sp. (Leclerc, 1815) were most common (> 50 %). High abundances were recorded in the Itupararanga Reservoir (44 161 ind./m^3) against the Broa Reservoir (3441 ind./m^3). Species such as *Asplanchna* sp. (77 886 ind./m^3), *Kellicotia bostoniensis* (14 043 ind./m^3), nauplius (29 941 ind./m^3), and copepodites of Cyclopoida (21 658 ind./m^3) were most abundant in the seven reservoirs.

At sampling sites, ZBI values ranged from 7.2 to 15.8 (Table 5). The Salto Grande and Broa reservoirs were shown to have poor water quality conditions, while the other reservoirs were classified as regular. For the ICZ, the reservoirs were classified as having poor water quality. Regarding validation, the ZBI showed significative correlation mainly with CLa ($r_S = 0.54$; $p = 0.01$), trophic state index ($r_S = 0.45$; $p = 0.03$), total phosphorus ($r_S = 0.33$; $p = 0.13$), and first principal component axis ($r_S = 0.43$; $p = 0.04$) (Fig. 2).

Table 4. Taxonomic list and respective optimum values (OP). *Lista taxonômica e respectivos valores de ótimo (OP).*

Taxa	OP	Taxa	OP
Calanoida		**Rotifera**	
Copepodito	15.86	*Anuraeopsis* sp. Lauterborn 1900	9.29
Nauplius	12.82	*Ascomorpha* sp. Harring, 1913	9.82
Notodiaptomus cearensis (Wright, 1936)	20.63	*Asplanchna brightwellii* Gosse, 1850	5.14
Notodiaptomus conifer (Sars, 1901)	26.73	*Asplanchna* sp. Eckstein, 1883	6.88
Notodiaptomus henseni (Dahl, 1894)	15.78	*Brachionus angularis* Gosse, 1851	8.52
Notodiaptomus iheringi (Wright, 1935)	16.69	*Brachionus caudatus* Barrois & Daday, 1894	5.98
Notodiaptomus sp. Kiefer, 1936	15.49	*Brachionus falcatus* Zacharias, 1898	11.63
Cyclopoida		*Brachionus mirus* Daday, 1905	5.16
Copepodito	8.15	*Brachionus plicatilis* Müller, 1786	16.16
Nauplius	7.35	*Brachionus* sp. Pallas, 1766	5.66
Mesocyclops longisetus (Thiebaud, 1912)	17.74	*Brachionus variabilis* Hempel, 1896	11.63
Mesocyclops ogunnus Onabamiro, 1957	26.73	*Collotheca* sp. Harring, 1913	10.13
Mesocyclops sp. Sars, 1914	12.60	*Conochilus unicornis* Rousselet, 1892	18.27
Metacyclops sp. Kiefer, 1927	13.75	*Epiphanes* sp. Ehrenberg, 1832	13v.75
Thermocyclops decipiens (Kiefer, 1929)	11.20	*Euchlanis* sp. Ehrenberg, 1832	8.45
Thermocyclops minutus (Lowndes, 1934)	7.41	*Filinia opoliensis* (Zacharias, 1898)	25.38
Thermocyclops sp. Kiefer, 1927	11.63	*Filinia* sp. Bory De St. Vincent, 1824	27.55
Cladocera		*Hexarthra* sp. Schmarda, 1854	26.73
Alona sp. Baird, 1843	26.73	*Kellicottia bostoniensis* (Rousselet,1908)	18.28
Bosmina freyi De Melo & Hebert, 1994	9.09	*Keratella americana* Carlin,1943	7.60
Bosmina sp. Baird, 1845	12.84	*Keratella cochlearis* (Gosse,1851)	18.32
Bosminopsis deitersi Richard, 1895	0.86	*Keratella quadrata* (Müller, 1786)	12.34
Ceriodaphnia cornuta (Sars, 1885)	7.07	*Keratella* sp. Bory De St. Vincent, 1822	6.60
Ceriodaphnia silvestrii Daday, 1902	10.21	*Keratella tropica* (Apstein, 1907)	18.02
Ceriodaphnia sp. Dana, 1853	11.30	*Lecane* sp. Nitzsch, 1827	6.41
Chydoridae	13.75	*Lepadella* sp. Bory de St. Vincent, 1826	7.64
Daphnia gessneri (Herbst, 1967)	5.98	*Polyarthra vulgaris* Carlin,1943	10.71
Daphnia sp. Müeller, 1785	8.82	*Pompholyx* sp. Gosse, 1851	10.08
Diaphanosoma sp. Fischer, 1850	4.89	*Ptygura* sp. Ehrenberg, 1832	13.51
Diaphanosoma spinulosum Herbst, 1975	19.34	*Synchaeta stylata* Wierzejski, 1893	6.01
Moina sp. Baird, 1850	12.57	*Trichocerca capucina* (Wierzejski & Zacharias, 1893)	5.51
		Trichocerca longiseta (Schrank, 1802)	29.74
		Trichocerca sp. Lamarck, 1801	11.84

DISCUSSION

Few studies have developed reservoir quality indices using zooplankton communities. Here, the species score method was differential. A linear response is the simplest approximation, whereas a unimodal response model assumes that species have an optimum in an environmental gradient (Leps & Smilauer, 2003; Jamil *et al.*, 2014). Therefore, algae biomass may be considered a good indicator of trophic gradients, mainly in reservoirs (Boyer *et al.*, 2009). The present study represents the first instance of a zooplankton trophic index using optimum values in Brazilian reservoirs. The method allowed us to predict the trophic status and showed good prospects due to good adherence with indicator variables.

Using the ZBI index, surface waters of the Salto Grande Reservoir were considered of poor quality. The results obtained by Dornfeld *et al.* (2006) for this reservoir showed an impacted environment that can cause adverse effects to biota, mainly because of the input of organic and inorganic (industrial) pollution. Although considered of poor quality, the Broa Reservoir was considered oligotrophic some years ago (Argenton, 2004). Despite the Rio Grande arm being considered to have regular quality, Mariani *et al.* (2008) and Pompêo (2017) highlighted the problems of algal bloom and the constant use of copper sulfate.

Since 2003, the São Paulo State Environmental Agency has used an ICZ based on zooplankton data and water quality diagram (CETESB, 2006). However, despite its simplicity, this index can present some problems. The ICZ considers the presence or absence of main zooplankton groups and uses a ratio of total number of calanoids (better water quality) and cyclopoids (poor quality). For calculation, three main taxa (Rotifera, Cladocera, and Copepoda) are required, but are not always present. Except in the Rio Grande arm, all reservoirs contain the above-mentioned groups. According to the ICZ, more than 50 % of the points had poor conditions, although some sampling points had regular water quality. Therefore, the ICZ results are observed to be close to the ZBI values.

It was not possible to use other environmental quality indices such conducted by some authors (Montagud *et al.*, 2019). The use of the Wetland Zooplankton Index (WZI) must be made with modifications for tropical regions since it has been developed for wetlands in the Great Lakes. These authors confirmed that further research is required to confirm its suitability for other regions and other vegetated habitats (Lougheed & Chow-Fraser, 2002). In Spanish reservoirs, researchers used a Zooplankton Reservoir Trophic Index with tolerance values. A total of 13 reservoirs (37.1 %) was considered to have good or superior status, 14 reservoirs (40 %) have moderate status, 6 reservoirs (17 %) have deficient condition, and 2 (5.7 %) are of poor quality (CHE, 2015). As mentioned above, the index was developed for reservoirs located in temperate regions.

Through linear regression analysis, Ejsmont-Karabin (2012) and Ejsmont-Karabin & Karabin (2013) established different formulas for predicting trophic gradients in Polish lakes. Parameters were verified since abundance and richness can be used as metrics. Biomass of Cyclopoida, Cyclopoida/Calanoida ratios and percentage of cyclopoid biomass were the most significant variables to include in trophic prediction models. Rotifer numbers, percentage of species indicative of high trophic levels in the indicative group's numbers, and percentage of tecta form in the population of *Keratella cochlearis* were the most significant parameters. Although regression coefficients may be used for different types of lakes, the formulas may be

Table 5. Water quality class obtained from different zooplanktonic indices. Legends: Salto Grande (SG), Barra Bonita (BB), Broa (BR), Itupararanga (IT), Atibainha (AT), Rio Grande (RG), and Igaratá (IG). River inlet (R), central (C, 2), dam zones (D), and water outlet (E). Zooplanktonic community index for reservoirs (ICZ), Zooplankton biotic Index (ZBI) e Trophic state index (TSI). *Classes de qualidade da água obtidos de diferentes índices zooplanctônicos. Legendas: Salto Grande (SG), Barra Bonita (BB), Broa (BR), Itupararanga (IT), Atibainha (AT), Rio Grande (RG) e Igaratá (IG). Entrada de água (R), centro (C,2), zona de barragem (D) e saída de água (E). Índice da comunidade zooplanctônica para reservatórios (ICZ), Índice biótico do Zooplâncton (ZBI) e, Índice de estado trófico (TSI).*

Sites	ICZ	ZBI	TSI	
SG-R	Poor	Poor (15.06)	69	Hypereutrophic
SG-C	Poor	Poor (14.39)	67	Supereutrophic
SG-D	Regular	Poor (14.05)	67	Supereutrophic
BB-R	Poor	Regular (9.59)	67	Supereutrophic
BB-C	Regular	Regular (9.18)	65	Supereutrophic
BB-D	Regular	Regular (8.49)	63	Eutrophic
BR-R	Regular	Poor (15.30)	65	Supereutrophic
BR-C	Regular	Poor (15.84)	64	Supereutrophic
BR-D	Regular	Poor (13.86)	66	Supereutrophic
IT-R	Poor	Regular (8.40)	62	Supereutrophic
IT-C	Poor	Regular (7.23)	59	Mesotrophic
IT-D	Poor	Regular (9.04)	60	Eutrophic
AT-R	Regular	Regular (9.24)	58	Mesotrophic
AT-C	Poor	Regular (8.83)	57	Mesotrophic
AT-C5	Poor	Regular (9.73)	55	Mesotrophic
RG-R	Poor	Regular (8.20)	61	Eutrophic
RG-C	Poor	Regular (8.90)	60	Eutrophic
RG-D	Poor	Regular (7.61)	58	Mesotrophic
IG-R	Regular	Regular (9.04)	50	Oligotrophic
IG-2	Regular	Regular (10.35)	54	Mesotrophic
IG-E	Regular	Regular (9.31)	56	Mesotrophic

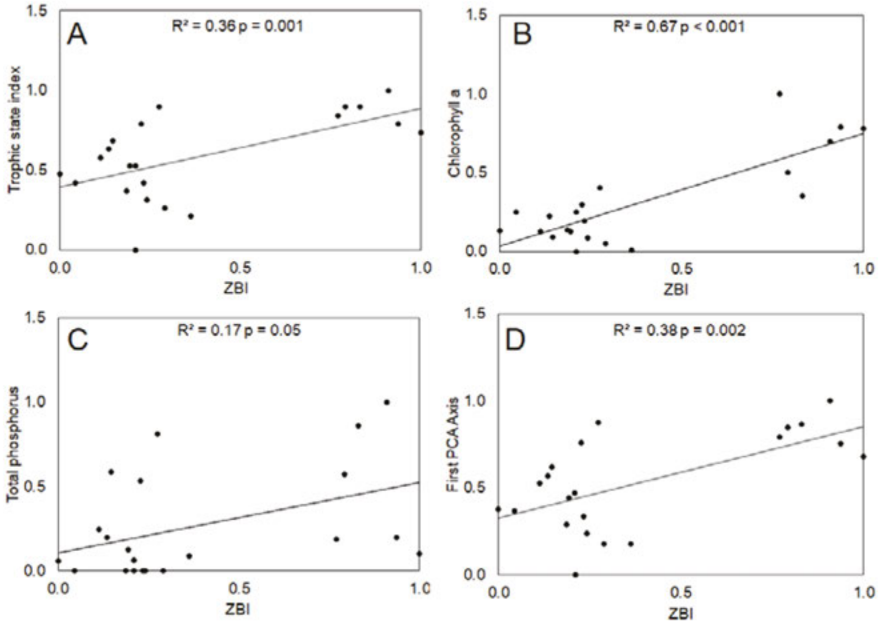

Figure 2. Relationship between standardized Zooplankton Biotic Index (ZBI) values and total phosphorus, chlorophyll a, trophic status index (TSI), and first principal component axis (PCA). Legends: Zooplankton Biotic Index (ZBI), determination coefficient (R^2), and significance values (p). All data were transformed for a better graph display. *Relação entre os valores padronizados do Índice Biótico do Zooplâncton (ZBI) e fósforo total, clorofila a, índice de estado trófico e o primeiro eixo do componentes principais (PCA). Legendas: Índice Biótico do Zooplâncton (ZBI), coeficiente de determinação (*R^2*) e valores de significância (p). Todos os dados foram transformados para uma melhor visualização no gráfico.*

useful only in preparing similar indices for lakes in central and northern Europe.

In a German lowland river, a plankton index of biotic integrity was developed to assess effects of human disturbances on the ecosystem. From 36 original metrics, only six were chosen to compose the index. The general ecological status of the region was considered as moderate regardless of seasonal variations, which was lower than the requirement (good status) of the WFD of 2015. The relatively lower ecological status was probably caused by point sources, diffuse source emissions and artificial drainage systems of the study area (Wu *et al.*, 2012). Through historical data analysis, Kane *et al.* (2009) developed a planktonic index of biotic integrity in the Great Lakes (Lake Erie). Discriminant analysis was used one of the differentials used to determine the best impact variables; in addition, combined zooplankton and phytoplankton data were also used. The results obtained showed that the increase in water quality score from 1970 to the mid-1990s, and its subsequent decline, reflected the changing trophic status of the lake. A differential of this study was the inclusion of two biological communities, allowing a better evaluation of the environmental integrity of the ecosystem. As another example, a zooplankton index of biotic integrity was developed for estuarine waters, using data from a long-term environmental assessment program. The choice metrics were the Simpson diversity index, abundance of barnacle larvae, rotifers, cladocerans, copepods, total mesozooplankton, and predators. The composite index of biotic integrity correctly classified approximately 94 % of the impaired samples and approximately 82 % of the reference samples. Average classification efficiency was 88 % (Carpenter *et al.*, 2006).

As observed, few studies have attempted to develop biological indices mainly with zooplankton. It should be noted that most studies were based on the multimeric index method (e.g. Karr, 1981, Hering *et al.*, 2006) and weighted values for each taxon were not calculated. In the present study, these values were calculated species by species and thus the index shows greater functionality. One of the main problems related to the use of a multimetric index is that the metrics do not always correlate with environmental variables. To elaborate the index, metrics such as total richness, abundance, and biomass must be used. After selection, the scale is calculated through analysis of percentile values. Both methods have advantages and disadvantages and the choice is the responsibility of the researcher.

When applying the proposed index (ZBI), we recommend that a few steps be followed. First, it is necessary to verify which species have a relationship with the eutrophication gradient (phosphorus, CLa, etc.). This assumption can be tested with a simple test of correlation. After analysis of the unimodal peaks, optimum and tolerance values should be calculated, and values that do not always coincide should be highlighted. For example, Sousa *et al.* (2008) investigated reservoirs in northeastern Brazil and calculated optimum and tolerance values taking into account eutrophication and salinization parameters. Using the values obtained in the present study, we tested equations proposed by other authors but could not verify good adherence.

CONCLUSION

The developed index proved to be efficient in estimating parameters related to eutrophication. It should also be noted that the Salto Grande and Broa reservoirs had poor water quality, while other reservoirs were classified as regular. In addition, the present study provides environmental weighted values for each species and can be applied to, and compared with, other tropical/subtropical reservoirs. As far as possible should be adapted the indices from other continents. For a better understanding of the environmental quality, the ecological indices derived from different aquatic communities should be integrated into a composite index.

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Macrophytes

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Phenology of macrophytes in coastal environments: *Utricularia australis* (R. Br.) and *Stuckenia pectinata* (L.) Börner in an interdunal pond within the Albufera de València Natural Park

Maria A. Rodrigo and Sara Calero

Integrative Ecology lab. Ecology, Ethology and Evolution (e3) group. Cavanilles Institute for Biodiversity and Evolutionary Biology, University of Valencia, Catedràtic José Beltran 2. E-46980-Paterna, València, Spain.

* Corresponding author: maria.a.rodrigo@uv.es

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ABSTRACT

Phenology of macrophytes in coastal environments: *Utricularia australis* (R. Br.) and *Stuckenia pectinata* (L.) Börner in an interdunal pond within the Albufera de València Natural Park

Macrophytes are key elements for biodiversity in Mediterranean ponds and coastal lagoons. To predict the long-term persistence of aquatic plant populations in a changing environment is essential to understand their phenology, i.e. the timing of ecological processes coupled with environmental factors. *Utricularia australis* and *Stuckenia pectinata* (=*Potamogeton pectinatus*) populations growing in a brackish interdunal pond within the Albufera de València Natural Park were monitored over three years. We describe their life-cycles under shallow conditions and relate them to accumulated heat and radiation. *U. australis* overwintered as turions; turion germination happened in February at 125-210 growing degree-days (GDD), at a daily mean temperature (DMT) of 10-11 °C, and with 150-220 hours of accumulated sunshine (h Sun). The flowering period started in June (2450 GDD, 26 °C DMT, 1365 h Sun), but only in 2014 and in the sunniest areas of the pond. The turion formation started in early August (3800-4000 GDD, 20-28 °C DMT, 1900-2180 h Sun). A reduction in *U. australis* biomass and coverage was observed throughout time. *S. pectinata* exhibited a perennial population restricted to the pond deeper basins. It initiated its life-cycle in late February-early March, mainly by re-sprouting from old stems and roots, vigorously producing new shoots with green leaves (400-440 GDD, 11-12 °C DMT, 300-380 h Sun). In early May, the production of the first spikes was detected (840-1045 GDD, 17-18 °C DMT, 820-900 h Sun). By mid-June, fertilization was complete and drupelet growth began (1430-1800 GDD, 20-23 °C DMT, 1190-1230 h Sun). Increased salinity and competition with charophytes developed in the pond, negatively affected the fertility and abundance dynamics of *S. pectinata*. *U. australis* performance declined with the reduction in free-water surface due to the expansion of helophytes, mainly cattails. The *S. pectinata* life-cycle in this pond fits in with the latitudinal pattern of a prolonged life-cycle with a delayed reproduction for southern populations. The restoration of degraded or destroyed aquatic ecosystems is a turning point for local populations to recover, but some management of the restored waterbodies is required to guarantee the preservation of peculiar and endangered species. *In situ* continuous monitoring of water temperature, and other relevant variables for submerged vegetation (such as light availability and salinity), is needed to have a more precise image of the environmental factor values required to pass from one phenological event to another, and for a better comparison among populations from different origins.

Key words: Aquatic angiosperms, Mediterranean ecosystems, carnivorous plant, life-cycles, growing degree-days (GDD)

RESUMEN

Fenología de macrófitos en ambientes costeros:* Utricularia australis *(R. Br.) y* Stuckenia pectinata *(L.) Börner en una laguna interdunar del Parc Natural de l'Albufera de València

Los macrófitos son elementos clave para la biodiversidad de lagunas del Mediterráneo. Para predecir la persistencia a largo plazo de las poblaciones de plantas acuáticas en un ambiente cambiante es esencial entender su fenología, i.e. el ritmo de los procesos ecológicos relacionados con los factores ambientales. Hemos estudiado durante tres años una población de Utricularia australis *y una de* Stuckenia pectinata *(*=Potamogeton pectinatus*) en una laguna interdunal salobre del Parc Natural de l'Albufera de València. Describimos sus ciclos vitales en condiciones someras y los relacionamos con el calor y la radiación acumuladas.* U. australis *pasó el invierno en forma de turiones; su germinación sucedió en febrero con 125-210 grados-día (GD), a una temperatura media diaria (TMD) de 10-11 °C y con 150-220 horas acumuladas de radiación solar (h Sol). La floración comenzó en junio (2450 GD/26 °C TMD/1365 h Sol), únicamente en 2014 y en las zonas más soleadas. La formación de turiones comenzó en agosto (3800-4000 GD/20-28 °C TMD/1900-2180 h Sol). Observamos una reducción en la biomasa y cobertura de* U. australis *a lo largo del tiempo.* S. pectinata *presentó una población perenne restringida a las cubetas más profundas de la laguna. Inició su ciclo vital a finales de febrero-principios de marzo, a partir de los rebrotes de los viejos tallos sumergidos (400-440 GD/11-12 °C TMD/300-380 h Sol). A comienzos de mayo detectamos las primeras flores (840-1045 GD/17-18 °C TMD/820-900 h Sol). Sobre mediados de junio la fertilización se completó y comenzó el crecimiento de los frutos (1430-1800 GD/20-23 °C TMD/1190-1230 h Sol). El aumento de salinidad y la competencia con los carófitos de la laguna afectó negativamente la fertilidad y la dinámica de* S. pectinata*. El crecimiento de* U. australis *cayó con la reducción de la superficie de agua libre por expansión de los helófitos. El ciclo de vida de* S. pectinata *en esta laguna se corresponde con el patrón latitudinal que atribuye ciclos de vida prolongados con reproducción tardía a las poblaciones de baja latitud. La restauración de ecosistemas acuáticos (destruidos-degradados) es fundamental para la recuperación de poblaciones locales, si bien es necesaria la gestión de estos ecosistemas para garantizar la preservación de especies peculiares y amenazadas. Se requiere el seguimiento* in situ *y continuo de la temperatura del agua y otras variables (disponibilidad luminosa, salinidad) para obtener una imagen precisa de los valores de los factores ambientales requeridos para pasar de una fenofase a otra, para así comparar entre poblaciones de origen diferente.*

Palabras clave: *Angiospermas acuáticas, ecosistemas mediterráneos, planta carnívora, ciclos de vida, grados-día*

INTRODUCTION

Ponds are the most widespread aquatic habitat, and collectively dominate the total area of continental standing waters in Europe; this being especially true for Mediterranean countries (Miracle *et al.*, 2010). Ponds also play many other valuable roles such as enhancing biodiversity, mitigating diffuse pollution or regulating temperature and humidity. Macrophytes are key elements for biodiversity in ponds and coastal lagoons of the Mediterranean (Chappuis *et al.*, 2012), one of the world's major biodiversity hot spots (Myers *et al.*, 2000), and they are of key interest for management and conservation purposes (Chappuis *et al.*, 2014).

Most aquatic angiosperms operate mixed reproductive strategies involving clonal and sexual reproduction, hence they disperse by both vegetative propagules and seeds (Philbrick & Les, 1996; Eckert *et al.*, 2016). This is the case of widely distributed species such as *Utricularia australis* R. Br. and *Stuckenia pectinata* (L.) Börner (=*Potamogeton pectinatus* L.). However, some shallow permanent waterbodies will turn into temporary ones under the drying situation predicted for the Mediterranean region (IPCC, 2014). Submerged macrophytes will be exposed to desiccation, and although plant fragments from some species are partially tolerant to it (Barnes *et al.*, 2013), the production of drought-resistant propagules will determine the persistence of these communities (Eckert *et al.*, 2016). Understanding the reproductive strategies, particularly the timing (phenology) of sexual reproduction, and the life-history patterns, including the types of propagules and their time of formation and germination/sprouting, is key for the conservation of submerged macrophytes and the ecosystems they inhabit.

Utricularia australis is a free-floating carnivorous species of aquatic bladderwort (Taylor, 1989). Its rootless shoots take up all necessary nutrients, either from the water or from captured prey (aquatic crustaceans, mites, nematodes, rotifers and protozoa) inside its traps or bladders. It usually grows in standing, nutrient-poor and humic waters. Its main life-history traits include extensive clonal reproduction, high dispersal ability of vegetative propagules (shoot fragments and turions), and rare-to-sporadic sexual reproduction (Adamec, 2011). *U. australis* is distributed in a vast geographic range in temperate and tropical regions, except North and South America (Taylor, 1989). There is no evidence that the species is threatened in any major parts of its range, and therefore it is listed by the IUCN as

"Least Concern" (Champion, 2014). Conversely, its status in the Mediterranean region is not as good as would be desired. In the Spanish territory, few littoral aquatic ecosystems have survived centuries of desiccation and destruction, and even fewer are free from pollution and eutrophication problems. For example, desiccation and drainage as well as water extraction and regulation, are the main causes of macrophyte loss in Catalan stagnant waters (Chappuis *et al.*, 2011). Thus, *U. australis* is catalogued as "in danger of extinction" in Andalusia, the Basque Country and the Valencian Autonomous Community, while it is considered as "vulnerable" in Asturias, Catalonia and Madrid (Anthos, 2017).

Stuckenia pectinata, commonly known as sago pondweed or fennel pondweed, is a perennial submerged macrophyte which grows abundantly within a wide distribution area (Kantrud, 1990). Some authors have proposed a latitude-correlated switch in its life-cycle strategy (Pilon *et al.*, 2002), although, in fact, little is known about *S. pectinata*'s reproductive ecology (Ganie *et al.*, 2016). The species employs several clonal (tubers, rhizomes, nodal plantlets, plant fragments) and sexual (fruits) modes of reproduction (Van Wijk, 1989). This highly flexible reproductive strategy contributes to the rapid spread of the species across different habitats.

Over three years, one population of *U. australis* and one of *S. pectinata* growing in a brackish shallow pond within the Albufera de València Natural Park (Spain) were monitored. The main objectives of this study are (i) to describe the different phenophases (i.e. the observable stages in the annual life cycle of an organism) of these two species in shallow coastal Mediterranean ecosystems, in coexistence with others macrophytes (charophytes and helophytes), under habitat interannual variation, and (ii) to explore how their phenological patterns respond or not to abiotic environmental variables. For the first time for these species, combined data of water daily mean temperature, accumulated heat and accumulated hours of sunshine for the precise time of the different phenological events are provided. The study also delves into the understanding of the sexual reproductive phenology of the species, by comparing our data from a lower-latitude ecosystem to the available information from other latitudes. Since the two-studied species are distributed world-wide, they constitute an excellent tool to analyse the phenological responses to environmental factors that vary with latitude.

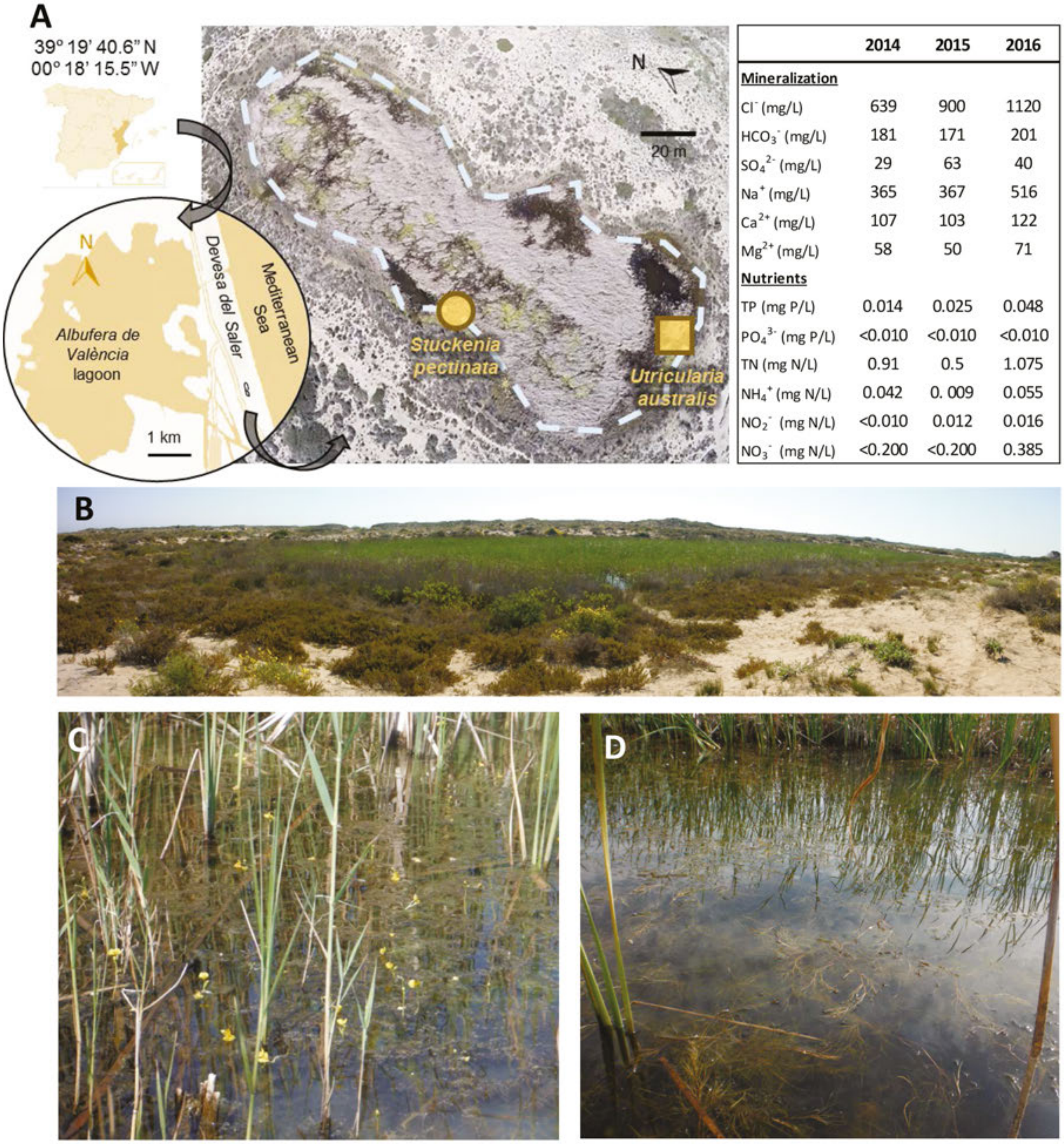
MATERIAL AND METHODS

Study area

The Albufera de València Natural Park is located on the east coast of Spain (Miracle & Sahuquillo, 2002). It includes one of the largest coastal lagoons in the Iberian Peninsula, separated from the Mediterranean Sea by a 14 km long and 1 km wide sand bar (Fig. 1A). This area, called *Devesa del Saler*, included several dune ridges, with a developed pine forest in the inner stable one, and a set of oligohaline shallow ponds. During the 1970s, an urbanization plan destroyed the seashore dune front and the dune slacks. Later, the area was protected (becoming a Natural Park in 1986; Natura 2000 network), and the regional authorities executed several restoration projects to reconstruct the former foredunes, their vegetation, and both temporary and permanent waterbodies. One of these ecosystems, the Llacuna Nova del Canyar (LNC, hereafter; Fig. 1) was created in 2007 as a shallow permanent pond with an approximate area of 5900 m^2. Most of the pond has a water level between 0.5 and 1 m, except for some deeper basins (1.5 m maximum depth).

Stuckenia pectinata (L.) Börner (=*Potamogeton pectinatus* L.) has been present in the pond since its reconstruction thanks to the work of the Natural Park managers, who planted some specimens from nearby waterbodies (J.M. Benavent, pers. comm.). *Utricularia australis* R. Br. was first detected in the LNC in October 2013 (Calero *et al.*, 2015). Both species formed the submerged vegetation community of the pond together with dense beds of *Chara hispida*, which occupied the shores and the deeper parts, and *Chara aspera* and *Nitella hyalina*, mainly located on the shores (Calero *et al.*, 2015, 2017). The pond held a high density of helophytic vegetation (Fig 1B; genera *Typha*, *Phragmites*, *Juncus* and *Scirpus*).

The LNC is an oligo-haline pond with a high concentration of chloride and sodium which



	2014	2015	2016
Mineralization			
Cl^- (mg/L)	639	900	1120
HCO_3^- (mg/L)	181	171	201
SO_4^{2-} (mg/L)	29	63	40
Na^+ (mg/L)	365	367	516
Ca^{2+} (mg/L)	107	103	122
Mg^{2+} (mg/L)	58	50	71
Nutrients			
TP (mg P/L)	0.014	0.025	0.048
PO_4^{3-} (mg P/L)	<0.010	<0.010	<0.010
TN (mg N/L)	0.91	0.5	1.075
NH_4^+ (mg N/L)	0.042	0. 009	0.055
NO_2^- (mg N/L)	<0.010	0.012	0.016
NO_3^- (mg N/L)	<0.200	<0.200	0.385

Figure 1. A: Location of the LNC within the coastal dune system of the Albufera de València Natural Park, and location of the sampling sites for *Utricularia australis* and *Stuckenia pectinata*. Chemical features of the LNC in 2014-2016. Analyses were performed in January 2014, and March 2015 and 2016. B: General view of the studied pond in spring 2015. C: Water surface of the shore of the interdunal pond covered by *U. australis* specimens, some of them flowered. D: The *S. pectinata* meadow in the interdunal pond. *A: Localización de la LNC en el sistema dunar costero del Parc Natural de l'Albufera de València, y ubicación de las zonas de muestreo de* Utricularia australis *y* Stuckenia pectinata*. Características químicas de la LNC en 2014-2016. Los análisis se realizaron en enero 2014, y en marzo 2015 y 2016. B: Vista general de la laguna estudiada en primavera de 2015. C: Superficie del agua en la orilla de la laguna interdunar cubierta por especímenes de* U. australis*, algunos de ellos en flor. D: Pradera de* S. pectinata *en la laguna.*

almost doubled from 2014 to 2016 (Fig. 1A). The water is nitrogen-poor; soluble reactive phosphorus concentrations were always lower than 0.01 mgP/L, and TP concentrations increased throughout time but remained below 0.05 mgP/L.

Collection of macrophytes and phenological data

The macrophyte samples were taken monthly, from January 2014 to December 2016, by sampling in the last week of each month, and on approximately the same day for the three years. During spring and summer, the sampling was bi-weekly for better detection of all phenophases throughout the expected time of the highest fertility.

Since *S. pectinata* distribution was mainly restricted to one of the deeper basins of the pond (Fig. 1D), this species was sampled only at this site where it coexisted with *Chara hispida*. The sampling was performed from the shore with the assistance of a hook. *U. australis* had a scattered distribution throughout the whole pond, but it was collected by hand at one shallow spot (< 50 cm deep) located in the southern part of the pond (Fig. 1C). The sampling effort was intended to be the same at each date and site (for *S. pectinata* site, the hook was thrown three times into the centre of the basin and all the specimens of both *S. pectinata* and *C. hispida* were collected; for *U. australis* site, specimens were randomly collected). For *S. pectinata*, 25-30 specimens by sampling date were collected on average. For *U. australis*, 40, 20 and 15 specimens were sampled, on average, by sampling date in 2014, 2015 and 2016, respectively (due to the reduction of its biomass; see Results). Fresh macrophyte material was placed in plastic bags and transported in a portable cooler to the Integrative Ecology Lab (ICBiBE, University of Valencia).

All plants were observed with a binocular loupe (Olympus SZ61, 67.5x maximum magnification) to accurately count and monitor the aspect and development stage of sexual and vegetative structures (flowers and fruits; also bladders and

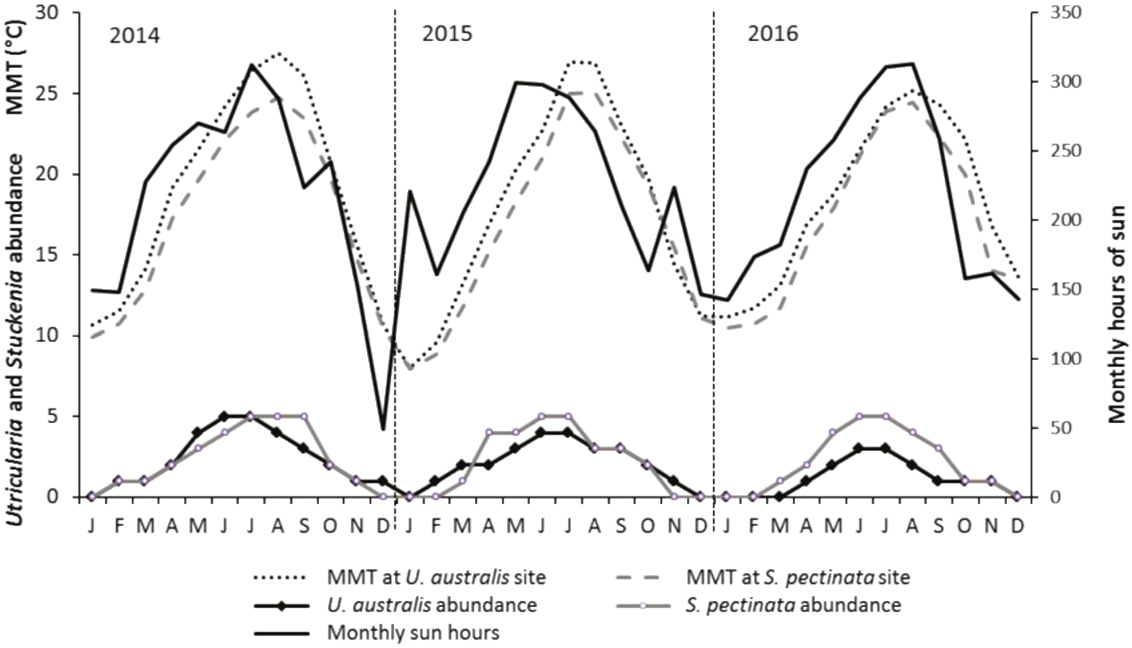


Figure 2. Dynamics of accumulated hours of sun per month, monthly mean temperature (MMT) at the two sites where the *U. australis* and *S. pectinata* were sampled and semi-quantitative (from 0 to 5) abundances of the two macrophyte species. *Dinámica del número de horas de sol por mes, temperatura media mensual en las zona de muestreo de* U. australis *y* S. pectinata *y abundancia semi-quantitativa (de 0 a 5) de las dos especies de macrófitos.*

turions for *U. australis*) in each specimen. The general aspect of *U. australis* was based mainly on the leaf colour, and four categories were considered: intense green, yellow-green, brown-green and senescent specimens. The phenological events considered were germination of turions, vegetative growth (abundance increase), flowering, formation of turions and senescence for *U. australis*, We distinguished two different phenological states for turions (Fig. S1, see Supplementary information available at http://www.limnetica.net/en/limnetica): (i) developing turions, already having a spherical dark green appearance, but strongly connected to the mother shoot (intense-green, yellow-green and brown-green specimens), and (ii) developed turions, with a darker brownish colour, easily detachable with forceps from the mother shoot (brown-green and senescent specimens). Two categories of bladder colour were considered: (i) purple, which is the colour of the anthocyanin from the plant tissues in an acidic medium, i.e. when the prey have not yet been digested, and (ii) dark blue-black, which indicates a basic medium due to the digestion of the animal proteins. The general aspect of *S. pectinata* was also based on the leaf colour and three categories were considered: green, brown and senescent specimens. For this species the phenological events considered were sprouting, vegetative growth, flowering and fruiting periods, as well as senescence.

Qualitative-semiquantitative estimation of macrophyte biomass and coverage

Due to the restrictions imposed by the environmental authorities concerning the collection of large samples of macrophytes from this protected pond (particularly for *U. australis*), biomass and distribution estimates are only qualitative/-semi-quantitative. These estimations are derived from in situ observations and photographs which were taken at the *S. pectinata* and *U. australis* sampling sites each sampling date. Moreover, the whole pond was checked to get an approximate idea of *U. australis* coverage and flowering, and additional photographs were taken. With this information, 6 categories of qualitative abundance/coverage were established (0: No specimens, 1: Very few specimens (< 10 % coverage), 2: Few specimens (10-40 %), 3: Medium abundance (40-70 %), 4: Abundant (70-90 %), 5: Highly abundant (> 90 %); (Fig. S2, see Supplementary information available at http://www.limnetica.net/en/limnetica). The photographs were also used to estimate the coverage of emergent vegetation in the *U. australis* site (Fig. S3, see Supplementary information available at http://www.limnetica.net/en/limnetica). The quantity of charophytes that appeared in the samplings with the hook when *S. pectinata* was sampled was used to roughly estimate the amount of charophytes in relation to *S. pectinata* in this basin.

Measurement of limnological variables

Two probes provided with data loggers (Onset HOBO; U20-001 model) were set up (submerged) in a shallow (*Utricularia* site) and a deeper (*Stuckenia* site) location in the pond to measure underwater temperature every half an hour. After subsequent data processing, daily mean temperature (DMT), monthly mean temperature (MMT) and growing degree-days (GDD) were calculated for the whole study period. GDD is a broadly applied parameter (Trudgill *et al.*, 2005) to measure the accumulation of heat throughout the year (from a starting point or biofix and above a baseline temperature) that allows a species to develop from one phenophase to the next in its life cycle. GDD were calculated using the HOBOware Pro Growing Degree Days Assistant. We used the Actual Temperature Method (no cut-off), which uses the logging interval of the temperature data, 30 minutes in our case, to perform a numerical integration. We considered 4 ºC as baseline temperature. The method described uses the area between the curve and this low threshold to compute the GDD. We used the 1st January as the starting date (biofix). Further information concerning the methodology can be found in Calero *et al.* (2017). Accumulated hours of sun by day (h Sun) were obtained from the European Climate Assessment and Dataset (Valencia station, number 237 –located 18 km from the pond-; Klein Tank *et al.*, 2002). Monthly hours of sun (MHS) were calculated to understand the synergistic roles of temperature and light in macrophyte phenology.

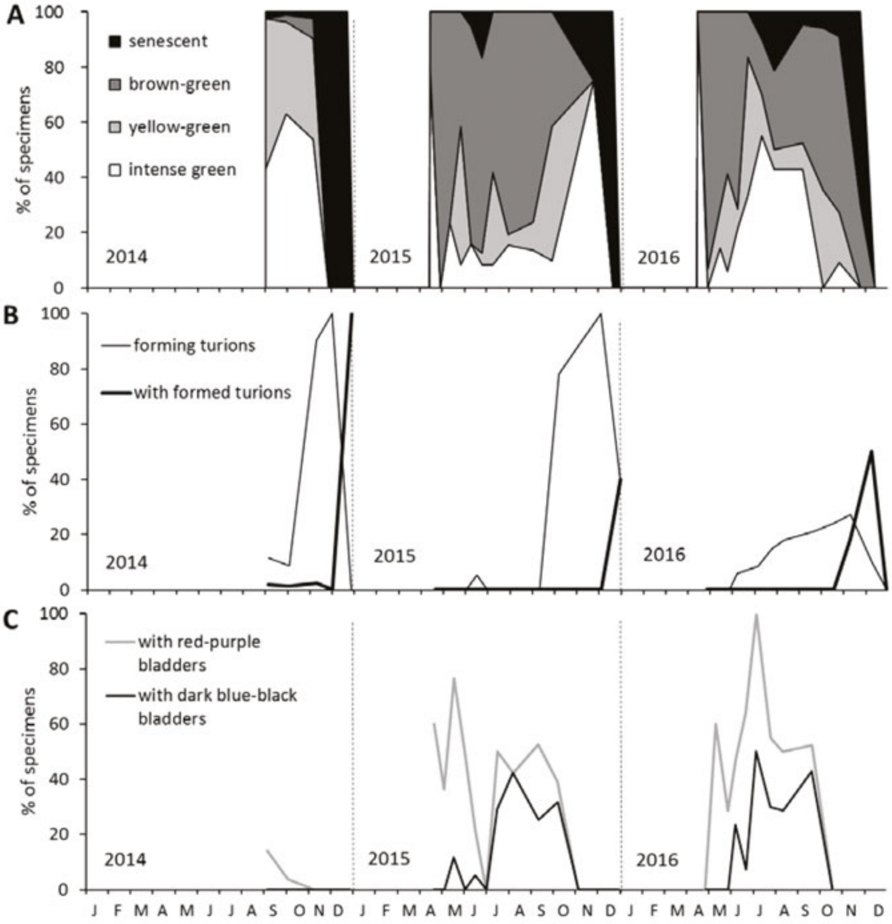


Figure 3. Phenology of some features of *U. australis* represented as the percentage of specimens showing the indicated characteristic throughout the study period. A: Changes in the colour of the plants (data registered from September 2014). B: Formation of turions. C: Presence of purple and dark blue-black bladders. *Fenología de algunas características de* U. australis *representadas por el porcentaje de especímenes que mostraban las características indicadas a lo largo del período de estudio. A: cambios en el color de las plantas. B: Formación de turiones. C: Presencia de utrículos púrpura y azul oscuro-negros.*

Salinity, pH and dissolved oxygen were measured monthly with portable field equipment (Multiline F/Set-3, WTW) at both sites. Water samples were taken monthly to measure sestonic chlorophyll *a* (according to Calero *et al.*, 2015), and annually to analyse total nitrogen (TN) and total phosphorus (TP) concentrations, as well as the solute composition of the water. The IIAMA laboratory (UPV) and the *Laboratorios Tecnológicos de Levante* performed these latter analyses using standard methods (APHA, AWWA, WEF, 2012).

Statistical analysis

One-way ANOVA tests for repeated measures were used to compare the means of the monthly number of hours of sun between 2014, 2015 and 2016, as well as for monthly mean temperatures in each macrophyte site. The normality of the distribution of the analysed variables and the homoscedasticity of samples were tested by the Kolmogorov–Smirnov and Levene tests, respectively. The salinity trend was analysed by means of linear regression. Two months (October 2015 and December 2016) with heavy rains were not

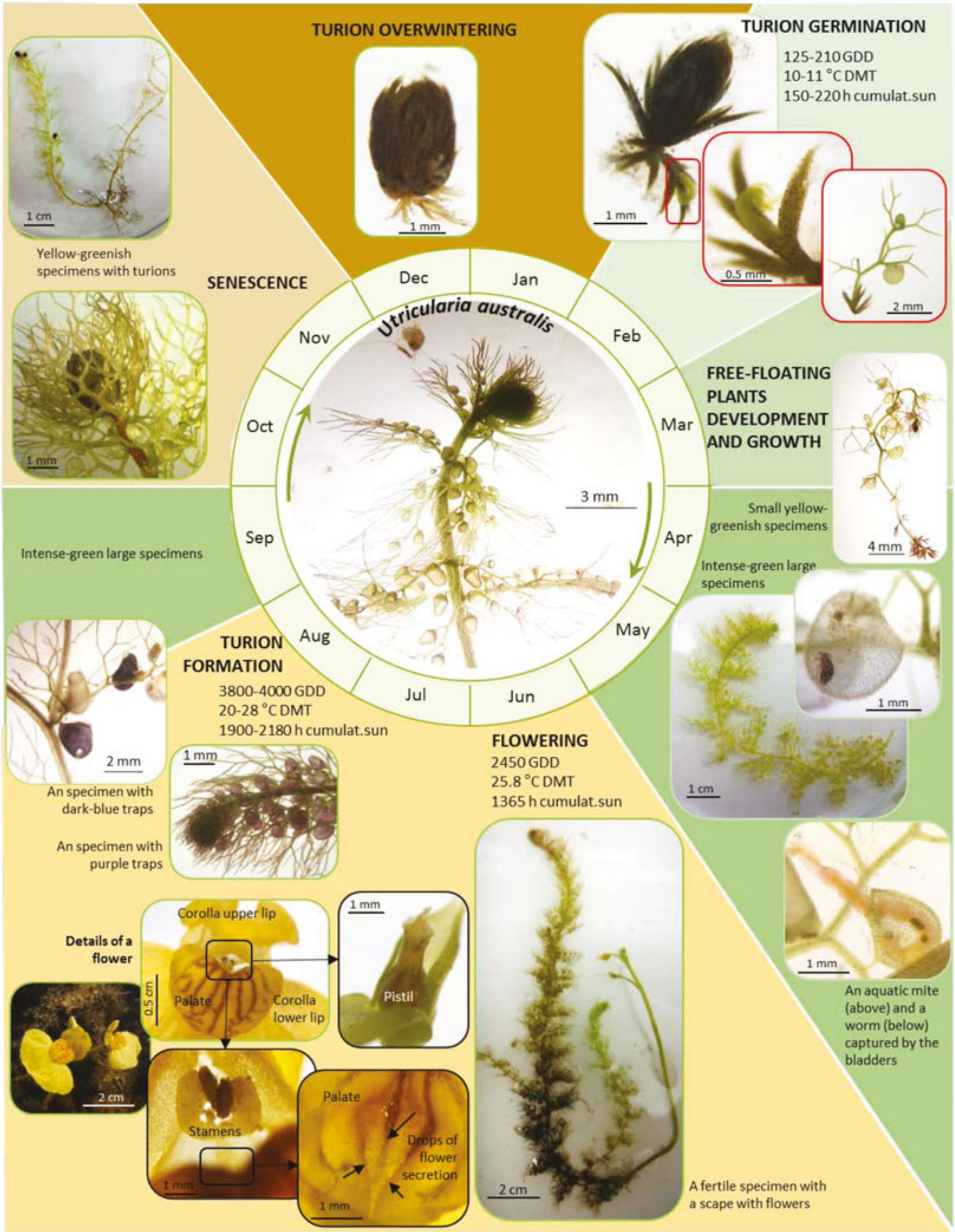


Figure 4. The life-cycle of the *Utricularia australis* population in the LNC. The different events are distributed throughout the months of the year in a circular diagram. Accumulated growing degree-days (GDD), water daily mean temperature (DMT) and accumulated sunshine (hours of sun) for the most important phenological events are shown. *El ciclo de vida de la población de* Utricularia australis *en la LNC. Los diferentes eventos están distribuidos a lo largo de los meses del año en un diagrama circular. Se muestran los grados-día acumulados, la temperatura del agua media diaria (DMT) y las horas acumuladas de radiación solar (horas de sol) para los eventos fenológicos más relevantes.*

considered in the analyses. To describe the relationship between macrophyte features (abundance and phenological events) and environmental data, stepwise multiple linear regression models were performed to test said relationships. Statistical significance was considered when $p < 0.05$. The IBM SPSS 19.0 statistical package was used to perform all statistical analyses.

RESULTS

Utricularia australis life-cycle

After the first detection in the pond of some specimens of *U. australis*, forming turions, in October-November 2013, no specimens were observed over the next two months. By February 2014, some free-floating specimens appeared

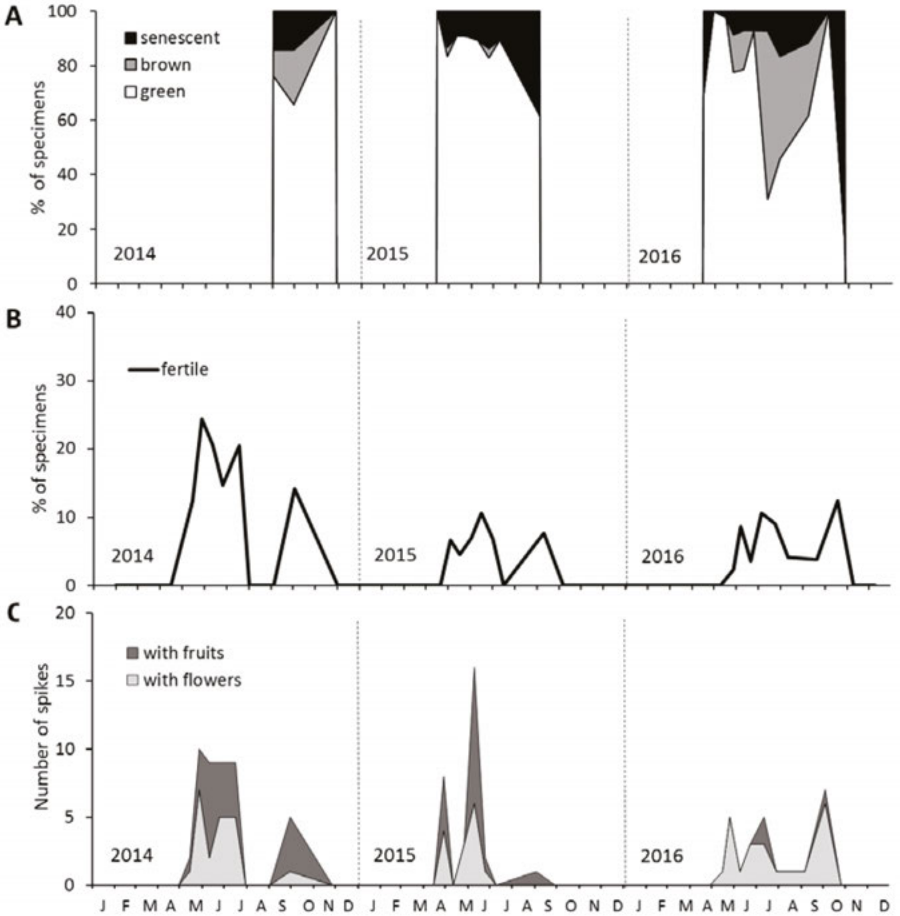


Figure 5. Phenology of some features of *S. pectinata* represented as the percentage of specimens showing the indicated characteristic throughout the study period. A: Changes in the colour of the plants (data registered from September 2014). “Green specimens” include specimens with bright green stem and leaves, but also others with brown stem and green leaves. “Brown specimens” include old plants with new sprouting leaves. B: Percentage of specimens bearing flowers and/or fruits. C: Number of spikes bearing flowers (being formed and already formed) and spikes bearing fruits in any state. *Fenología de algunas características de* S. pectinata *representadas como el porcentaje de especímenes que muestran la característica indicada a lo largo del período de estudio. A: Cambios en el color de las plantas (datos obtenidos a partir de septiembre 2014). “Especímenes verdes” incluye aquellos especímenes con tallos y hojas verdes brillantes pero algunos también con tallos oscuros y hojas verdes. “Especímenes marrones” incluye plantas viejas con nuevas hojas. B: Porcentaje de especímenes portadores de flores y/o frutos. C: Número de tallos portadores de flores (que se están formando o ya formadas) y tallos portadores de frutos en cualquier estado.*

(Fig. 2). The maximum population abundance occurred between June and July. The specimen colour changed from yellow-green, intense green to brown-green at the end of the season (Fig. 3A). While a high biomass of free-floating plants covered most of the water surface of the pond, flowering was restricted to its southern part, except for some isolated specimens that flowered on the eastern shore. The flowering period started in June 2014, lasting until August (Fig. 4; Table S1, Supplementary information available at http://www.limnetica.net/en/limnetica). By then, the first specimens developing turions on the tip of the shoot appeared, as well as others with developed turions (Fig. 3B). The formation of turions was observed until November, when 100 % of specimens were senescent. In 2015, the abundance peaked again between June and July. The formation of turions started one month later than in the previous year. In 2016, there was a clear reduction in abundance (Fig. 2); the first specimens were not detected until April, and some shoots already began to form turions in May. No flowering occurred in 2015 or 2016.

The plants exhibited a large quantity of bladders, on many occasions containing small animals which had been captured such as aquatic mites and worms (Fig. 4). In 2014, 14 % of the specimens possessed purple bladders at the beginning of September (Fig. 3C). This percentage largely increased in 2015 and 2016 up to 70-100 %. Dark blue-black bladders were also observed during these years in up to 40-50 % of specimens at the end of June-beginning of July.

In terms of accumulated heat and radiation (Fig. 4; Table S1, Supplementary information), the turions germinated at 125-210 GDD, 10-11 ºC DMT and 150-220 h Sun. The flowering period started at 2450 GDD, 25.8 ºC DMT and 1365 h Sun. The turion formation started at 3800-4000 GDD, between 20 and 27.8 ºC DMT and 1900-2180 h Sun.

Stuckenia pectinata life-cycle

In 2014, the overwintering old stems of *S. pectinata* started to vigorously produce new green leaves by the end of February, reached the maximum biomass in July-September and then rapidly decreased in October, when the shoots moved into the senescence stage (Fig. 2, 5A). In 2015 and 2016, shoots started to grow one month later, in March; the maximum biomass, lower than the first year, was achieved earlier. The percentage of senescent plants particularly increased in 2016 (Fig. 5A).

The reproductive period started at the end of April-beginning of May in the three years, with the highest percentage of fertile specimens in 2014, up to 25 %. In 2015 and 2016, the percentage of fertile shoots was similar (6-7 %; Fig. 5B). In 2016, the proportion of fertile shoots with fruits was very scarce (Fig. 5C). The fructification peak took place in mid-June in 2014 and 2015.

In terms of accumulated heat and radiation (Fig. 6; Table S1, Supplementary information), the re-sprouting from old stems in late February-early March took place at 400-440 GDD, 11-12 ºC DMT and 300-380 h Sun. In early May, the production of the first spikes was detected at 840-1045 GDD, 820-900 h Sun, and when the DMT was 17-18 ºC. By mid-June, fertilization was complete and drupelet growth began. At this time, the accumulated heat was 1430-1800 GDD and there was a DMT of 20-23 ºC and 1190-1230 h Sun.

Limnological variables

The monthly mean temperatures (MMT) (Fig. 2) between the three studied years were not statistically different in any of the macrophyte sites (repANOVA $F_{2\text{-}33} = 3.3$; $p = 0.060$ for the *Utricularia* site; repANOVA $F_{2\text{-}33} = 1.9$; $p = 0.170$ for the *Stuckenia* site). Nor was there interannual variability in the mean values of monthly hours of sun (MHS) (Fig. 2) (repANOVA $F_{2\text{-}33} = 0.44$; $p = 0.650$). Water salinity significantly increased over the three-year period (Fig. 7; $R^2 = 0.70$, $p < 0.001$ for the *Stuckenia* site; $R^2 = 0.24$, $p = 0.003$ for the *Utricularia* site). The trend of increased salinity was more remarkable in the *Stuckenia* site, where salinity changed from mean values of 1.7 g/L in 2014 to 2.5 g/L in 2016. The sestonic chlorophyll concentrations did not show any summer peak, and were below 20 µg/L most of the time in the *Utricularia* site (Fig. 7A). In the *Stuckenia* site, however, there was a constant pattern over the three year period: very low concentrations of chlorophyll during the cold months, but peaks in

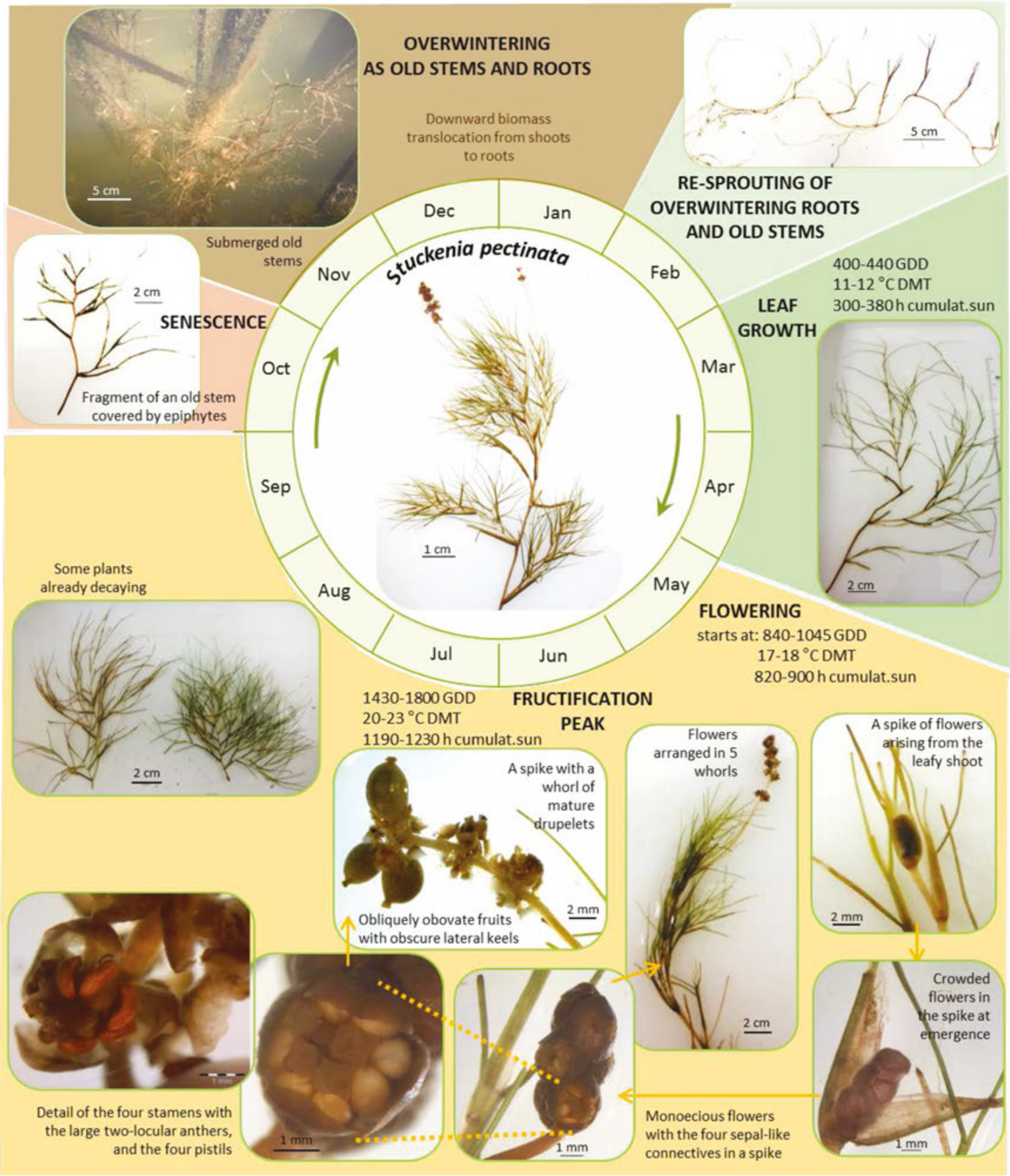


Figure 6. The life-cycle of the *Stuckenia pectinata* population in the LNC. The different events are distributed throughout the months of the year in a circular diagram. Accumulated growing degree-days (GDD), water daily mean temperature (DMT) and accumulated sunshine (hours of sun) for the most important phenological events are shown. *El ciclo de vida de la población de* Stuckenia pectinata *en la LNC. Los diferentes eventos están distribuidos a lo largo de los meses en un diagrama circular. Se muestran también los grados-día acumulados, la temperatura del agua media diaria (DMT) y las horas acumuladas de radiación solar (horas de sol) para los eventos fenológicos más relevantes.*

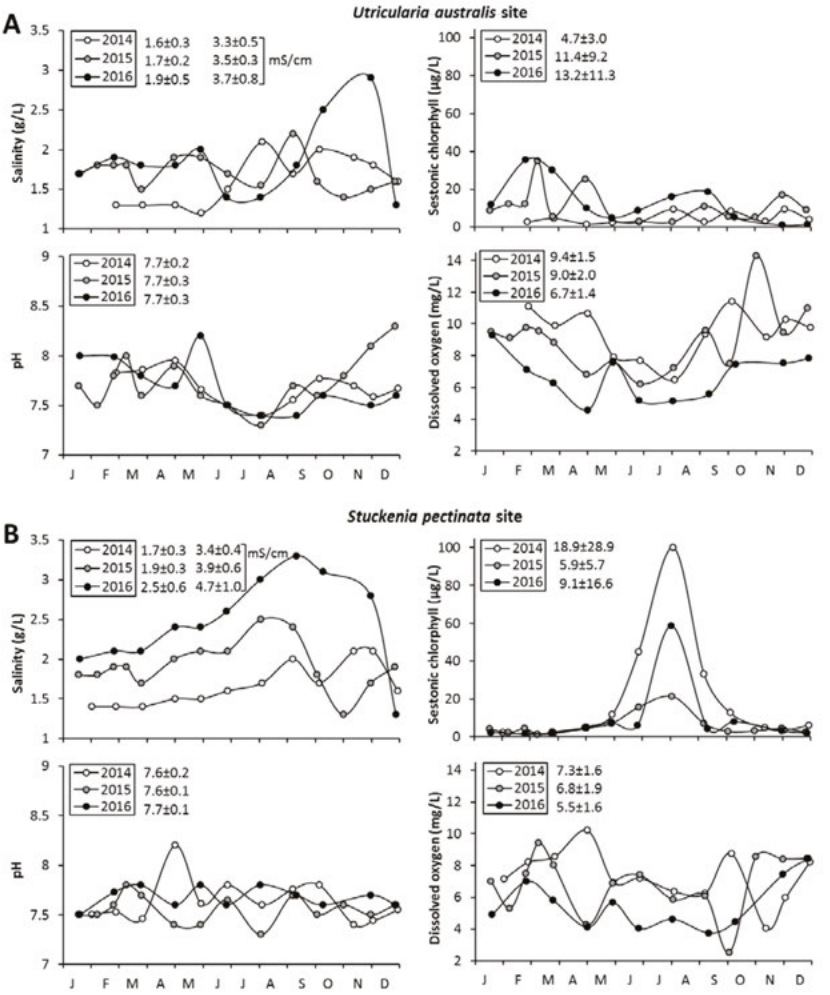


Figure 7. Fluctuations of limnological variables in the three consecutive years in the LNC. A: *Utricularia australis* site; B: *Stuckenia pectinata* site. Annual means±standard deviations are shown for each variable and each site. In the salinity graph, the annual mean (±SD) values for conductivity also appear. *Fluctuaciones de las variables limnológicas en los tres años consecutivos en la LNC. A: En la zona de* Utricularia australis*; B: En la zona de* Stuckenia pectinata*. Se muestran las medias anuales±desviaciones típicas para cada variable y en cada lugar. En la gráfica de salinidad, también aparecen las medias anuales (±DT) de la conductividad.*

the summer; the highest concentrations being found in 2014 (Fig. 7B). Water pH was very similar in both sites, with annual means of 7.6-7.7. Mean dissolved oxygen concentrations were quite variable at both sites, but always higher in the *Utricularia* site (Fig. 7A and 7B).

Multiple regression analyses of macrophyte features and the abiotic environment

The abundance of both *U. australis* and *S. pectinata* was only related to MMT and MHS (Table 1). Water salinity, pH and dissolved oxygen did not show any significant relationship. Turion formation in *U. australis* was not significantly related to either temperature or salinity. However, there was a significant negative relationship with MHS. The fertility of *S. pectinata* was significantly related to MMT and negatively related to water salinity (Table 1).

DISCUSSION

The life-cycle of *Utricularia australis* in the pond

After the arrival of *U. australis* in the LNC in autumn 2013, it overwintered by forming turions (Calero *et al.*, 2015), typical of the species (Taylor, 1989). When the turions began to germinate, the abundance of free-floating plants rapidly increased across the pond in spring 2014, despite being a nutrient-poor habitat. This situation was possible due to several ecophysiological adaptations that this plant exhibits, such as a very high net photosynthetic rate, its carnivorous activity, an efficient nutrient re-utilization from senescent shoots, and a very high affinity for mineral nutrient uptake from water (Adamec, 2011). The carnivorous activity of this *U. australis* population was proven not only by the presence of animals inside the traps, but also by the change in the colouring of the bladders, which varied throughout the annual cycle from purple to dark-blue, which is indicative of the digestion of the animal proteins (Cirujano *et al.*, 2014).

Even though sexual reproduction is quite uncommon for *U. australis* (Aguilella *et al.*, 2009; Araki & Kadono, 2003) this species flowered in the LNC during the summer of 2014, as we had already reported in Calero *et al.* (2015). The flowering species were very large and ramified, equivalent to the lower size threshold for sexual reproduction described by Adamec (2011), 40-70 cm in length depending on the system. The population from the LNC invested in sexual and vegetative reproduction at the same time. In August 2014, still in the flowering period, the specimens started to form turions; this pattern of starting with turion formation when the temperature was still high was repeated over the next two years. As shown by the negative relationship between the percentage of specimens forming turions and the monthly hours of sun, which explains more than 50 % of the variance in turion production, it seems that this process is more influenced by the length of the day. Our observations support the idea that solar radiation is a key factor for the development of *Utricularia* species (Englund *et al.*, 2003). The presence of turions, more than sexual reproduction, has allowed the persistence of this species for at least three consecutive years in the pond.

The sites where the population from the LNC flowered (the sampling site in the southern part of the pond as well as some spots in the eastern part) received high exposure to the sun due to the scarce growth of cattails. During 2015-2016, there was no repetition of either flowering or such massive vegetative growth, showing how the same population of *U. australis* may flower, or not, depending on the interannual variations of the habitat. We consider that the species found the optimal habitat for the allocation of resources in generative reproduction (Adamec, 2011; Englund *et al.*, 2003) in this pond during 2014, but not afterwards. Since we did not find any significant relationship between *U. australis* and water salinity, and taking into account that this species is considered to be a eurytopic species with a very wide ecological tolerance to water chemistry (Adamec, 2011), it seems that the increase in salinity experienced by the pond is not the reason for the observed decrease in biomass and coverage. Charophyte meadows, possible competitors of *U. australis*, did not grow significantly in the sampled site. However, the cattail surface greatly expanded from 2014 to 2016 (see Fig. S3, Supplementary information), particularly in the shallow areas of the shores, considerably reducing the solar radiation reaching the water surface as well as the spatial habitat for *U. australis* development. We consider that the decrease in *U. australis* biomass and coverage, and the lack of further flowering, is closely related to this reduction in the free-water surface throughout the pond.

The life-cycle of *Stuckenia pectinata* in the pond

The *S. pectinata* population from the LNC showed a perennial life-cycle which initiated in late February, mainly by rapid outgrowth of old plants that vigorously produced new shoots with green leaves. *S. pectinata* has been described to employ habitat-specific reproductive strategies (Van Wijk, 1989; Wan *et al.*, 2016). Sexual fruits, tubers and nodal plantlets are the most important reproductive propagules in standing waters (Ganie *et al.*, 2016). In the LNC, the main observed remains of *S. pectinata* during the cold season were old stems, these being the primary mode of vegetative reproduction and playing a key role in the annual regeneration. *S. pectinata* from the LNC also invested in sexual reproduction through the formation of flowers and achenes. However, the allocation of resources to sexual reproduction was not too high, since less than 25 % of the specimens in 2014, and less than 10 % in 2015 and 2016, were fertile. Other studies reported how *S. pectinata* can flower abundantly, but the significance of seed production for yearly survival is considered to be very limited (Van Wijk, 1989). In the LNC, the high density of charophytes (Calero *et al.*, in preparation) may have hindered the germination of seeds, the sprouting of tubers, and even the growth of small plantlets from the pond bottom.

S. pectinata has been described to grow luxuriantly in many aquatic habitats (Eid *et al.*, 2013). Its growth in the LNC, though, is quite limited

Table 1. Results of the stepwise multiple linear regression analyses performed on the abundance of the two macrophytes species, and on turion formation in *U. australis* and *S. pectinata* fertility taking into account the abiotic environmental variables. *: significant; **: highly significant. *Resultados de los análisis de regresión múltiple realizados sobre la abundancia de las dos especies de macrófitos y sobre la formación de turiones en* U. australis *y la fertilidad en* S. pectinata *usando las variables abióticas ambientales. *: significativo; **: altamente significativo.*

	Coeff.	R^2	Multiple^2	*p*
Utricularia australis				
Abundance				
Monthly Mean Temp.	0.15	0.66	0.661	0.001**
Monthly Hours of Sun	0.01	0.52	0.694	0.042*
Water Salinity	0.26	0.03	0.696	0.564
Water pH	-0.16	0.15	0.696	0.836
Water Dissol. O_2	0.12	0.07	0.715	0.171
Turion formation				
Monthly Hours of Sun	-0.50	0.51	0.508	0.005**
Monthly Mean Temp.	-0.65	0.28	0.523	0.730
Water Salinity	-20.00	0.00	0.552	0.303
Stuckenia pectinata				
Abundance				
Monthly Mean Temp.	0.19	0.74	0.737	<0.001**
Monthly Hours of Sun	0.01	0.69	0.819	0.001**
Water Salinity	-0.28	0.12	0.821	0.497
Water pH	-0.10	0.01	0.822	0.928
Water Dissol. O_2	-0.05	0.15	0.823	0.650
Fertility				
Monthly Mean Temp.	1.64	0.43	0.428	0.001**
Water salinity	-5.73	0.002	0.540	0.025*
Monthly Hours of Sun	-0.04	0.22	0.570	0.273

and its presence is restricted to the deeper basins. In one such basin, the cover and biomass of *S. pectinata* was lower in 2015 and 2016 than in 2014. Although the species has a high tolerance to water conductivity changes (Menéndez & Sánchez, 1998), fertility decreased over the three years, along with a gradual increase in salinity; higher salinity values also negatively affected *C. hispida* reproduction in a nearby pond (Calero *et al.*, 2018). Thus, we consider that the salinity increase observed in the LNC, particularly in this site, together with the massive coverage increase in charophytes (Calero *et al.*, in preparation) were the main causes of the *S. pectinata* reduction in performance throughout time. Hidding *et al.* (2010) reported how Chara's ability to act as a nutrient sink may contribute, through macronutrient-mediated competition, to the decline of *S. pectinata* in shallow lakes. In fact, in the present study, *S. pectinata* reached its maximum biomass after the other aquatic plants (*Typha domingensis* and *C. hispida*) which have their peak growth in summer. This can have a significant survival value to avoid competition for light and nutrients (Eid *et al.*, 2013).

Latitudinal variation of life-cycles

Several studies have reported latitudinal variation in the life-cycle of aquatic species, particularly in marine macro-algae, seagrasses and charophytes (Peters & Breeman, 1993; Calero *et al.*, 2017). However, information about latitudinal responses in freshwater angiosperms is not so abundant. For *U. australis*, there is an extensive bibliography concerning different aspects of its biology, but almost no information is available regarding its phenology (Thor, 1988); this fact prevents comparisons with our data. For *S. pectinata*, conversely, several studies focus on the phenology, biomass production and reproductive allocation at different locations. Based on information from Western Europe, Pilón *et al.* (2002) proposed a latitude-correlated switch in the *S. pectinata* life-cycle strategy: while northern populations show a short life-cycle, with an early and high investment in tuber biomass, low latitude populations have a prolonged life-cycle with delayed reproduction, an increased total plant biomass, but an earlier biomass peak. The *S. pectinata* life-cycle in the southern LCN fits in with this pattern; although the biomass peak was observed later than in other southern European sites, there was interannual variability among the three years under consideration. The differences in *S. pectinata*'s phenology between studies in Spanish lagoons (Menéndez & Comín, 1989; Menéndez *et al.*, 2002; Obrador *et al.*, 2007; Prado *et al.*, 2013, etc.), and in many other sites (Van Wijk, 1988, 1989; Pilon *et al.*, 2002, etc.) may be due to intrinsic (i.e. genetic) and extrinsic factors such as local abiotic factors (temperature, solar irradiance, length of growing season, day length, water light extinction, water depth, nutrient condition, etc.) and local biotic factors (competition and/or herbivory). Santamaría (2002) found, regarding irradiance, that latitudinal variation was less important than local and seasonal differences. Thus, we need simultaneous studies across latitudes with special emphasis on standardizing the samplings and analysing important variables such as GDD, cumulative sunshine, etc., to draw geographical patterns.

On the conservation of macrophytes

The case of *U. australis* is a good example of the situation of many aquatic plants in Spain. Although initially the species do not appear to have difficulties regarding their preservation, since they reproduce asexually and have a rapid development, the loss of habitats has endangered their populations. In the Albufera de València Natural Park, *U. australis* was formerly cited in several aquatic ecosystems, but the species was not found in any cited locality during the survey performed in 1995, nor in the surveys carried out from 1999 (Aguilella *et al.*, 2009). Thus, the discovery of the population in the LNC (Calero *et al.*, 2015) represented the reappearance of the species after an absence of more than 20 years in the Park. During the samplings, we observed that waterfowl, mainly ducks and herons, regularly visited the ponds. Thus, the establishment of the population likely occurred by means of waterfowl dispersion (a fragment on the legs, plumage and/or guts; Figuerola *et al.*, 2005). This new population of *U. australis* proves that the restoration of destroyed or degraded aquatic ecosystems is a turning point for local populations to recover, particularly for those endangered species in the Iberian Peninsula. In the studied waterbody, some management was necessary to reduce the extension of emergent vegetation, which seems to be the main cause of the regression of *U. australis*. Local authorities mechanically removed part of the helophytic biomass in November 2016, however, the emergent vegetation re-occupied most of the water surface by the next year (pers. observ.; October 2017). The removal of cattails to recover large populations of *U. australis* in this coastal pond is expensive and not sustainable in the long term. Therefore, maintaining small populations in different ponds, ensuring connectivity, appears to be the best option to recover populations of *U. australis* in the Natural Park.

S. pectinata reproduced mainly asexually in the LNC, but allocated some resources to flowering and fruit formation. This investment in sexual reproduction could assure the population's resistance to desiccation, a predicted trend for the ponds and coastal lagoons of the Mediterranean region (IPCC, 2014), as well as the genetic variability needed for a more likely adaptation of the species facing a changing environment. In this context, we highlight the need to continuously monitor water temperature and other relevant variables in situ, such as light availability and water salinity, in the long-term to better understand the environmental factors determining the reproduction of submerged vegetation, and its timing.

As addressed here, exploring or inferring relationships between biotic and abiotic variables in the short-term helps to disentangle which environmental factors to follow in long-term monitoring programs for macrophyte populations. Thanks to accurate knowledge of the conditions (the values of parameters such as accumulated degree-days and daily/monthly mean temperatures) that are required to pass from one phenological event to another, we will be able to compare among populations from different origins and across biogeographic ranges. All this information will eventually enable the prediction of the long-term persistence of species facing desiccation.

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Macroinvertebrates

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Role of plant architecture on littoral macroinvertebrates in temperate and subtropical shallow lakes: a comparative manipulative field experiment

Juan M. Clemente[1], Thomas Boll[2], Franco Teixeira-de Mello[1], Carlos Iglesias[1], Asger Roer Pedersen[3], Erik Jeppesen[2,4] and Mariana Meerhoff [1,2,*]

[1] Departamento de Ecología y Gestión Ambiental, Centro Universitario Regional del Este, Universidad de la República, Maldonado, Uruguay.
[2] Department of Bioscience, Aarhus University, Silkeborg, Denmark.
[3] Hammel Neurorehabilitation Centre and University Research Clinic, Aarhus University, Hammel, Denmark.
[4] Sino-Danish Centre for Education and Research, Beijing, China.

[*] Corresponding author: merluz@fcien.edu.uy

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ABSTRACT

Role of plant architecture on littoral macroinvertebrates in temperate and subtropical shallow lakes: a comparative manipulative field experiment

We dedicate this paper to the late Maria Rosa Miracle, who was a key researcher in the shallow lakes community, always enthusiastically dedicated to science, and always ready to discuss and guide younger scientists and fellow colleagues on the dynamics of zooplankton as well as on shallow lakes functioning at large. She will be sadly missed.

Plant habitat complexity can buffer against predator-prey interactions by offering physical refuges, and also novel microhabitats for alternative prey. In shallow lakes, submerged and free-floating plants affect predator-prey interactions with expected differences related to climate-driven differences in fish community structure. We tested the overall hypothesis that fish predation shapes the structure of plant-associated macroinvertebrate communities. By introducing artificial free-floating and submerged plants in a total of 14 shallow lakes under contrasting climate regimes (temperate and subtropical), we predicted that higher densities of macroinvertebrates would occur in the temperate zone where littoral fish predation is comparatively weaker than in the subtropics. We also tested the hypothesis that different structural complexities and % PVI would lead to different association patterns. Taxonomic richness, diversity, and densities of plant-attached macroinvertebrates were several fold higher in the temperate lakes. Macroinvertebrate densities per unit of plant weight were generally higher on the more complex free-floating than on the more abundant (as % PVI) submerged plants. The structure of littoral macroinvertebrates showed no clear relationship with the trophic state gradient. Fish communities, whose structure (biomass, density and habitat use) differed consistently between the temperate and subtropical locations, seemingly shaped the macroinvertebrate communities, most likely via predation and in the subtropics potentially also by competition. Free-floating plants appeared as a better anti-predation refuge or a preferred habitat for macroinvertebrates, particularly in the subtropics, but with increasing water turbidity this pattern became less distinct. In contrast, in the temperate lakes, the use of artificial plants by macroinvertebrates was rather related to the biomass of periphyton than to their refuge capacity, thus suggesting stronger bottom-up effects. A stronger fish predation pressure, which is to be expected with climate warming and eutrophication, may reduce the richness and abundance of plant-associated macroinvertebrates, with potentially strong impacts on shallow lake functioning. A warming-driven expansion of free-floating plants could, if moderate, weaken such effects, but a more diverse macrophyte community would facilitate co-existence of macroinvertebrate and fish.

Key words: habitat complexity, space for time substitution, artificial plants, free-floating plants, fish predation

RESUMEN

Rol de la arquitectura de las plantas sobre los macroinvertebrados litorales en lagos someros templados y subtropicales: una comparación experimental de campo

La complejidad del hábitat puede modular interacciones entre depredadores y presas al ofrecer refugio físico y nuevos microhábitats para competidores potenciales o presas alternativas. En los lagos someros, las plantas sumergidas y flotantes afectan las interacciones depredador-presa, con diferencias relacionadas a diferencias en la comunidad de peces influenciadas por el clima. Mediante la introducción de plantas flotantes libres y plantas sumergidas artificiales en un total de 14 lagos someros en dos regímenes climáticos contrastantes (templado y subtropical), analizamos la hipótesis de que la estructura de la comunidad de macroinvertebrados litorales está principalmente determinada por la presión de depredación de los peces. Por lo tanto, esperamos encontrar mayores densidades en la zona templada, donde la depredación por parte de los peces litorales es comparativamente más débil que en la zona subtropical. También analizamos la hipótesis de que las diferencias en complejidad estructural (mayor en las flotantes), así como en volumen ocupado por plantas (mayor en las sumergidas) promueve diferentes patrones de asociación en los macroinvertebrados.

La riqueza taxonómica, la diversidad y la densidad de los macroinvertebrados litorales fueron varias veces mayores en los lagos templados. Las densidades de macroinvertebrados (por unidad de masa de las plantas) fueron en general mayores en las plantas flotantes libres. La estructura de los macroinvertebrados litorales no mostró una relación clara con el gradiente de estado trófico estudiado. Los peces, cuya biomasa, densidad y uso del hábitat difirieron de forma consistente entre ambas zonas, determinaron la estructura de los macroinvertebrados probablemente vía depredación y en la zona subtropical potencialmente también por competencia. Las plantas flotantes libres parecieron actuar como un refugio más efectivo o hábitat preferido particularmente en la zona subtropical, pero con el aumento de la turbidez del agua el patrón se hizo menos evidente. En los lagos templados, el uso del hábitat estuvo más vinculado a la biomasa de perifiton, sugiriendo la ocurrencia de controles ascendentes. Una mayor presión de consumo por los peces (esperada con el calentamiento climático y un aumento de la eutrofización), puede reducir la riqueza y abundancia de los macroinvertebrados litorales con impactos potencialmente fuertes sobre el funcionamiento de los lagos someros. Una expansión de las plantas flotantes libres promovida por el calentamiento climático, podría, si fuera moderada, debilitar estos efectos. Sin embargo, una comunidad diversa de macrófitas seguramente promovería la coexistencia entre macroinvertebrados y peces.

Palabras clave: *complejidad de hábitat, sustitución tiempo por espacio, plantas artificiales, plantas flotantes libres, depredación por peces*

INTRODUCTION

Our understanding of lake functioning advanced dramatically with the recognition that the littoral-benthic and pelagic food chains are often very strongly linked (e.g. Timms & Moss, 1984; Vadeboncoeur *et al.*, 2002; Schindler & Scheuerell, 2002). By feeding in the littoral or benthic habitats, macroinvertebrates can transform and translocate organic matter and nutrients that ultimately reach fish and other top predators (Jones & Waldron, 2003), thus impacting whole-lake energy dynamics.

Habitat complexity in the different lake zones may affect whole ecosystem processes, not least by modulating predator-prey interactions. Macrophytes may reduce the feeding efficiency of fish and serve as a refuge for macroinvertebrates against fish predation (Diehl & Kornijóv, 1998) and for small and juvenile fish against piscivorous fish (Persson & Crowder, 1998). Particularly in vegetation-free areas (Leppä *et al.*, 2003), fish predation can reduce the density and biomass of benthic macroinvertebrates and alter community structure by reducing the density of the larger and more motile taxa (Mittelbach, 1988; Diehl & Kornijów, 1998). The predation pressure of fish on macroinvertebrates can lead to trophic cascades that ultimately reach periphyton and macrophytes (Brönmark & Vermaat, 1998), potentially influencing the environmental state (e.g. clear or turbid waters) of shallow lakes (Jones & Sayer, 2003; Burks *et al.*, 2006). Areas with macrophytes, therefore, often support a much higher biomass and diversity of macroinvertebrates than vegetation free areas (Hargeby *et al.*, 1994; Boll *et al.*, 2012). Besides acting as a refuge against predation, macrophytes support the growth of epiphyton that serves as an important food source for grazing macroinvertebrates

(Jones *et al.*, 1998). Oligochaetes and chironomids can substantially reduce epiphytic biomass (Cattaneo, 1983), but especially snails facilitate the growth of submerged macrophytes by removing epiphytes (Brönmark & Vermaat, 1998; Jones *et al.*, 1998).

The physical structure or architecture of macrophytes determines several components of habitat complexity and therefore influences the abundance and community structure of plant-associated macroinvertebrates. Their abundance is often higher on submerged than on floating-leaved (Cattaneo *et al.*, 1998) and emergent macrophytes (van de Meutter *et al.*, 2008). Besides, macrophytes with finely divided leaves often host a higher abundance of macroinvertebrates than macrophytes of simpler structure (Cheruvelil *et al.*, 2002; Xie *et al.*, 2006). Differences in macroinvertebrate densities between plant types may partly be explained by epiphyton production, because epiphyton biomass often increases with increasing plant complexity (Cattaneo *et al.*, 1998; Hao *et al.*, 2017).

The role of macrophytes for macroinvertebrates, not least their interaction with potential predators, has been elucidated in much more detail in shallow temperate lakes (Brönmark & Vermaat, 1998; Jones & Sayer, 2003, Boll *et al.*, 2012) than in subtropical and tropical lakes (Meerhoff *et al.*, 2007a; Thomaz *et al.*, 2008; Teixeira-de Mello *et al.*, 2016). However, a differential role of macrophyte architecture in several biotic interactions has been associated with climate-related differences in fish community structure. In (sub)tropical lakes, fish, particularly small species and individuals, occur in high abundances and are especially associated with submerged macrophytes (Meerhoff *et al.*, 2007a; Teixeira-de Mello *et al.*, 2009). Consequently, the refuge effect of submerged plants for zooplankton (Burks *et al.*, 2006), for instance, is substantially less efficient in the subtropics (Meerhoff *et al.*, 2007 b; González-Sagrario & Balseiro, 2010) and Mediterranean lakes (Tavsanoğlu *et al.*, 2015) than in similar lakes in the temperate zone.

Despite the importance of the effects produced by the physical structure of aquatic plants, no standardized methods to measure plant complexity exist, limiting comparisons among different plant species and ecosystems (reviewed in Kovalenko *et al.*, 2012). The use of artificial plants with controlled physical complexity can help decrease natural variability and allow for more specific hypothesis testing. Although plastic plants can promote different colonization patterns from those occurring on natural plants, typical confounding factors can be limited by the introduction of the same habitat modules along a trophic state gradient.

Here, we explored how plant-associated macroinvertebrates were affected by plant architecture, submerged or free-floating, in shallow lakes under different environmental scenarios, in particular along a gradient in trophic state and contrasting climates (temperate, Denmark, and subtropical, Uruguay).

We tested the hypothesis that the fish predation pressure shapes the structure of plant-associated macroinvertebrate communities. We expected to find impoverished littoral macroinvertebrate assemblages in lakes with high fish

Table 1. Main limnological characteristics of the lakes where the habitat modules were introduced, showing mean and range values for the temperate and subtropical zones (n = 7 in each country). *Principales características limnológicas de los 14 lagos donde se introdujeron los hábitats experimentales con plantas artificiales. Se muestran la media y el rango de los valores para los lagos templados y los lagos subtropicales (n = 7 en cada país).*

	Area (ha)	T (°C)	SD (m)	pH	TN µg/l	TP µg/l	Chl *a* µg/l
Temperate	18.2	17.4	0.96	7.4	915.7	47.4	30.6
	3--73	12--20.7	0.25--1.5	6.5--8.4	340--2090	14--76	2--40
Subtropical	20.2	21.7	0.8	7.3	735.4	63.8	19.7
	3--60	20.7--29	0.3--1.6	5.9--7.8	249--835	21--182	0.8--20

biomass, most pronouncedly in the subtropical lakes given the stronger predation pressure expected there (Meerhoff *et al.*, 2007a; 2012). We also predicted differences in the number and diversity of macroinvertebrates between both plant types, with outcome depending on the relative importance of complexity (higher for the free-floating plants) and the volume occupied by the plants (higher for the submerged plants). However, within each climate zone we expected macroinvertebrate density to increase with increasing nutrient concentrations and potentially increasing food availability (Blumenshine *et al.*, 1997; Diehl & Kornijów, 1998), despite variations in fish densities.

MATERIALS AND METHODS

Design and sampling methodology

Based on previous information, we paired a set of seven shallow temperate lakes (Denmark: 55 º-57 º N) varying along a trophic gradient (turbidity plus total phosphorus concentration) with a set of similar subtropical lakes (Uruguay: 30 º-35 º S). The lakes were paired according to a series of limnological characteristics (i.e. lake area, mean depth, Secchi disk, macrophyte % PVI, total phosphorus (TP), total nitrogen (TN), phytoplankton chlorophyll *a* (chl *a*), suspended solids, light attenuation, and pH; Table 1). We then ordered the lake pairs along a proxy trophic gradient determined by decreasing water transparency and nutrient concentrations (1-7; seven of the 10 lake sets in the study of fish by Teixeira-de Mello *et al.* (2009) were included in our study on macroinvertebrates) (Table 1).

Habitat modules resembling submerged and free-floating plant beds (four replicates of each) were introduced in a sheltered and plant-free littoral area of each lake (water depth: 1 meter). The artificial submerged plants and artificial roots of the floating plants consisted of the same plastic material (a total of 80-100 m of garlands made of finely dissected 3.0-cm long pieces per module). The artificial 0.80-1.0-m long submerged plants roughly mimicked the overall structure of *Elodea* spp., while each artificial free-floating plant consisted of a plastic disc (15 cm in diameter, 40 per module) to which a total of 2 m garlands were attached in pieces so as to roughly mimic the root network of water hyacinth, *Eichhornia crassipes* (Mart) Solms (for photos see Meerhoff *et al.*, 2007b). Local plant volumes inhabited were 49 % and 30 % for submerged and free-floating plants, respectively. Following Tokeshi & Arakaki (2012), habitat complexity is interpreted as all the different characteristics of structure, thus including spatial scale, size, density, spatial arrangement, and diversity (heterogeneity) of structural elements in an ecosystem. The artificial plants used in this study shared spatial scale, size, and density and varied in two of these complexity components (i.e. spatial arrangement and heterogeneity (length of) structural elements). We used the same habitat modules in both countries after they had been thoroughly rinsed, washed using high water pressure, disinfected with concentrated chlorine solution, and sun-dried.

Sampling campaigns were conducted in summer, four weeks after the introduction of the habitat modules, to allow periphyton and invertebrates to colonize the artificial plants (January 2005 in Uruguay and July 2005 in Denmark; average water temperatures were 25.5 and 18.1 ºC, respectively). Water samples for the analysis of TP, TN (Valderrama, 1981; Søndergaard *et al.*, 1992, respectively), chl *a* (Jespersen & Christoffersen, 1987), and alkalinity were collected from the open water of the lake, where also *in situ* measurements of Secchi depth, oxygen, pH, temperature, and conductivity (with Horiba field sensors) were conducted. Periphyton biomass was determined as chlorophyll *a* from a 10-cm long piece of one artificial plant or artificial root taken randomly from each habitat module at 10-20 cm depth from the surface.

Fish and large-bodied low-numbered macroinvertebrates were collected during the night with a cylindrical net (diameter 1.20 m, mesh size 0.3 cm), which was placed on the sediment under the habitat modules in the previous afternoon. We approached the modules in a boat and lifted the net in a quick maneuver using a 1.5-m-long stick with a hook. The nets, with the plant modules inside, were removed from the water and carefully inspected. We used an over-dose of anesthetic 2-phenoxi-ethanol to euthanize the fish (methodology suggested by the Honorary Animal Research Commission of Uruguay), which were afterwards identified, counted, weighed, and measured (standard length) (Teixeira-de Mello *et al.*, 2009). We used quantitative fish data from Teixeira-de Mello *et al.* (2009). We sampled the plant-associated macroinvertebrates (> 500 µm) during the day by carefully removing one artificial plant from each of the habitat modules. Macroinvertebrates were stored in 70 % ethanol and in the laboratory determined to genus level, whenever possible, using different identification keys (e.g. Merritt & Cummings, 1984). We estimated macroinvertebrate densities as individuals per weight of artificial plant, with the exception of densities of large-bodied (low-numbered) macroinvertebrates, which were included as individuals per m^2 of area occupied by plants. We assigned the identified individuals to broad trophic classification according to the literature and measured subsamples of chironomid larvae from all genera under microscope to determine their body size range.

Statistical analyses

Evenness (*J*, Pielou's evenness) and diversity (*H*', Shannon diversity) were calculated at the genus level using the formulae: $J = H' / \log N$ and $H' = - \sum(p_i * \log p_i)$, where p_i is the fraction of genus *i* in the sample (n_i / N). The individuals that could not be identified to genus level were included in the calculations of total density and as a single genus (per family) in the diversity, Pielou's evenness, and richness calculations. This conservative method assured that diversity was not boosted by the occurrence of a few individuals that could not be determined to genus level. All dependent variables, except diversity, evenness, and richness, were log(x)-transformed to achieve normality in the dataset. Diversity and evenness were x^2-transformed before analyses, and richness was untransformed.

We tested the effects of location (two levels), plant type (two levels), and a proxy of trophic state (seven levels) with a three-factor ANOVA. For *post hoc* analyses, we used Tukey HSD tests with the Tukey-Kramer adjustment. Dependent

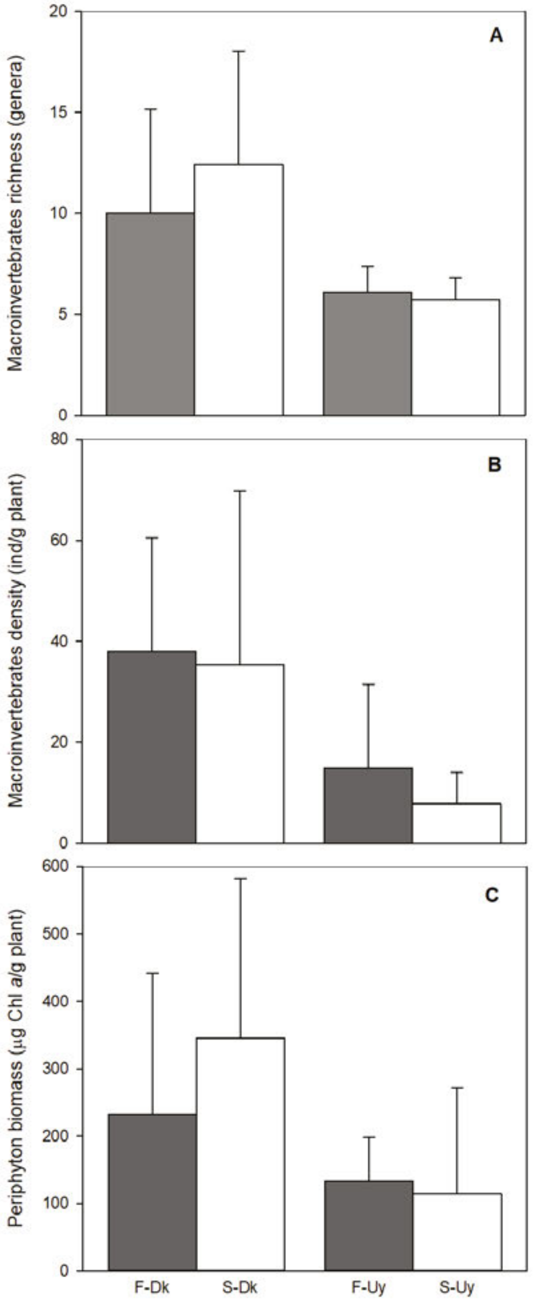

Figure 1. Mean richness of genera (A) and densities (B) of macroinvertebrates, and periphyton biomass (C) in both artificial plant types introduced in temperate (Dk) and subtropical (Uy) shallow lakes. F = free-floating plants, S = submerged plants. Data show the average of lake averages and associated standard deviations. *Riqueza de géneros (A) y densidades (B) promedio de macroinvertebrados y biomasa de perifiton (C) en ambos tipos de plantas artificiales introducidas en lagos someros templados (Dk) y subtropicales (Uy). F = plantas flotantes libres, S = plantas sumergidas. Los datos muestran el promedio de los promedios de cada lago y el desvío estándar asociado.*

variables included densities of macroinvertebrates per unit of plant biomass (g artificial plant), diversity, evenness and genera richness of macroinvertebrates, and periphyton biomass.

To facilitate interpretation of the potential habitat use by macroinvertebrates under the different environmental conditions, we estimated the density ratio between the two plant types (i.e. Submerged/Free-floating (hereafter S/F) density ratio). The S/F density ratio was calculated for densities of macroinvertebrates per gram of plant, and differences between the two countries were analyzed with one-way ANOVA. Since the overall statistical power was too low to include both countries in the same model, we analyzed variations in the S/F density ratio within each country in relation to lake concentrations of chl *a*, TP and TN, and Secchi disc depth, as well as in relation to mean fish biomass and periphyton biomass as indicators of potential predation pressure and food availability per lake, respectively (i.e. averaging data from the two plant types in each lake). The variables were log10-transformed before the analyses and tested in simple and multiple linear regressions with stepwise selection retaining variables significant at the 0.05 level (Stepwise selection with *p*-enter: 0.25 and *p*-leave: 0.05).

RESULTS

We found clear differences between the two climate zones in the structure of the macroinvertebrate communities associated with the artificial plants, in terms of taxonomic richness, density, and size structure (Fig. 1 A, B). Diptera was the dominant group in terms of abundance in both countries. Mollusks appeared with higher richness in the temperate lakes than in the subtropical lakes (where only the family Ampullaridae was found). In contrast, large-bodied motile macroinvertebrates, such as apple snails (*Pomacea canaliculata* Lamarck), shrimps (*Palaemonetes* cf. *argentinus* Nobili), and crabs (*Aegla* sp.), were only found in the subtropical lakes. Apple snails were most abundant in lakes with low nutrient levels. Crabs were only found in the three lakes with the highest fish biomass, while shrimps, the most abundant of the large macroinvertebrates, were absent or very rare in the most eutrophic lakes. Mean (± SD) densities were 2.1 ± 2.6, 14.0 ± 24.2, and 57.5 ± 90.3 ind./m^2 for apple snails, crabs, and shrimps, respectively, on the free-floating plants, and 6.4 ± 9.3, 19.4 ± 4.2, and 103.6 ± 122.6 ind./m^2, respectively, on the submerged plants. Amphipods (*Hyalella* sp. in Uruguay and *Gammarus* spp. in Denmark) were found in the lakes with the lowest potential predation pressure, whereas the proportion of oligochaetes rose with increasing fish biomass (with the exception of the Danish lake at trophic level 5, where dipterans were the overall dominant group despite a relatively high fish biomass).

We found a significantly higher richness among the artificial plants in the temperate lakes (see statistical results in Table 2). A total of 36 families and 68 genera occurred there, whereas 19 macroinvertebrate families and 35 genera occurred among the artificial plants of the subtropical lakes (Fig. 1A). We found a total of 19 genera of chironomids (four of which were predators) among the plants in the temperate lakes and only seven in the subtropical lakes. Mean taxonomic diversity was higher in the temperate lakes (1.87 ± 0.37 and 1.13 ± 0.26 (mean ± SD) in the Danish and Uruguayan lakes, respectively, Table 2). In contrast to diversity, evenness did not differ significantly between the two climate zones (Pielou's evenness = 0.77 ± 0.09 and 0.64 ± 0.14; mean ± SD, in the Danish and Uruguayan lakes, respectively).

Mean density of macroinvertebrates was also significantly higher in the temperate lakes (36.6 ± 27.9 and 11.4 ± 12.6 ind./g plant in Denmark and Uruguay, respectively) (Fig. 1B). The size range of chironomids was broad in the temperate lakes (from 0.5 to 19.0 mm), whereas most chironomids in the subtropical lakes had a much narrower body size range (13.0-15.0 mm). Considering macroinvertebrate functional groups, we found ca. 8-fold higher densities of predators, 10-fold higher densities of grazers, and 2-fold higher densities of collectors in the temperate lakes (average of plant types) than in the subtropical lakes.

Regarding our overall hypothesis, we found that macroinvertebrate density was significantly and negatively related to fish biomass in the subtropics, whereas no such relationship occurred

Table 2. Effects of location (climate zone, C), proxy of trophic state (L), and plant type (P) on littoral macroinvertebrate structure and periphyton biomass, indicating the degrees of freedom (D.F.) and the F and *p*-values in the three-factor ANOVA tests. Densities of macroinvertebrates per biomass of plants (ind./g of plant) and periphyton biomass (µg/g of plant) were log(x) transformed before analysis. Richness is number of genera. *Efectos de la zona climática (C), proxy del estado trófico (L) y tipo de planta (P) sobre la estructura de los macroinvertebrados litorales y biomasa de perifiton, indicando los grados de libertad (D.F.) y los valores de F y* p *de las pruebas ANOVA de 3 vías. Las densidades de macroinvertebrados por biomasa de plantas y la biomasa de perifiton por biomasa de plantas fueron transformadas mediante* log_{10} *antes de los análisis. La riqueza refiere al número de géneros.*

	Density (ind. g/ plant)			**Richness**		
Explanatory factors	*D.F.*	*F value*	*p*	*D.F.*	*F value*	*p*
Climate zone (C)	1,71	227.83	<0.0001	1,71	361.63	<0.0001
Plant Type (P)	1,71	24.09	<0.0001	1,71	1.53	0.2200
Trophic Level (L)	6,71	13.31	<0.0001	6,71	31.24	<0.0001
C*P	1,71	0.69	0.4079	1,71	5.84	0.0182
C*L	6,71	34.38	<0.0001	6,71	34.88	<0.0001
P *L	6,71	3.64	0.0033	6,71	1.92	0.0890
C*P* L	6,71	5.11	0.0002	6,71	5.36	0.0001

	Diversity (H')			**Periphyton biomass**		
Explanatory factors	*D.F.*	*F value*	*p*	*D.F.*	*F value*	*p*
Climate zone (C)	1,71	229.32	<0.0001	1,77	70.90	<0.0001
Plant Type (P)	1,71	1.28	0.2615	1,77	2.13	0.1488
Trophic Level (L)	6,71	11.81	<0.0001	6,77	9.20	<0.0001
C*P	1,71	0.00	0.9891	1,77	35.14	<0.0001
C*L	6,71	6.85	<0.0001	6,77	16.32	<0.0001
P *L	6,71	1.59	0.1630	6,77	3.31	0.0060
C*P* L	6,71	2.93	0.0131			

in the temperate lakes ($R^2 = 0.70$ $p = 0.018$ and $R^2 = 0.03$ $p = 0.695$, respectively).

Plant type did not significantly affect diversity and evenness of macroinvertebrates, although a few more families were found on the submerged than on the free-floating plants in both climate zones. However, the overall densities of macroinvertebrates were higher on the free-floating than on the submerged plants (per gram of artificial plant) in most lakes (Table 2). In the Danish lakes, differences were insignificant as we found an average density of 37.9 ± 22.5 and 35.3 ± 34.4 (mean ± SD) ind./g plant among the free-floating and the submerged plants, respectively, whereas in the Uruguayan lakes we found 14.9 ± 16.5 and 7.9 ± 6.2 ind./g plant among the free-floating and submerged plants, respectively.

Periphyton biomass was significantly higher in the temperate than in the subtropical lakes (Table 2; 288.7 ± 222.7 and 123.6 ± 116.3 µg chl-*a*/g plant, in Denmark and Uruguay, respectively). Free-floating plants in the subtropics supported a higher biomass of periphyton per unit of weight than did the submerged plants, the opposite being observed in the temperate lakes (significant country x plant type interaction, Table 2) (232.5 ± 209.2 and 345.4 ± 237.2 (mean ± SD µg chl-*a*/g plant) for free-floating and submerged plants in Denmark and 133.2 ± 64.6 and 114.0 ± 157.8 µg chl-*a*/g plant for free-floating and submerged plants, respectively, in Uruguay, Fig. 1C).

Plant type had a differential effect on macroinvertebrate density under different environmental conditions in the lakes in the two countries (significant third order interaction in the ANOVA test, Table 2) despite the fact that the mean S/F density ratio did not differ significantly between the countries (one-way ANOVA $F_{1,12} = 2.71$; $p = 0.125$).

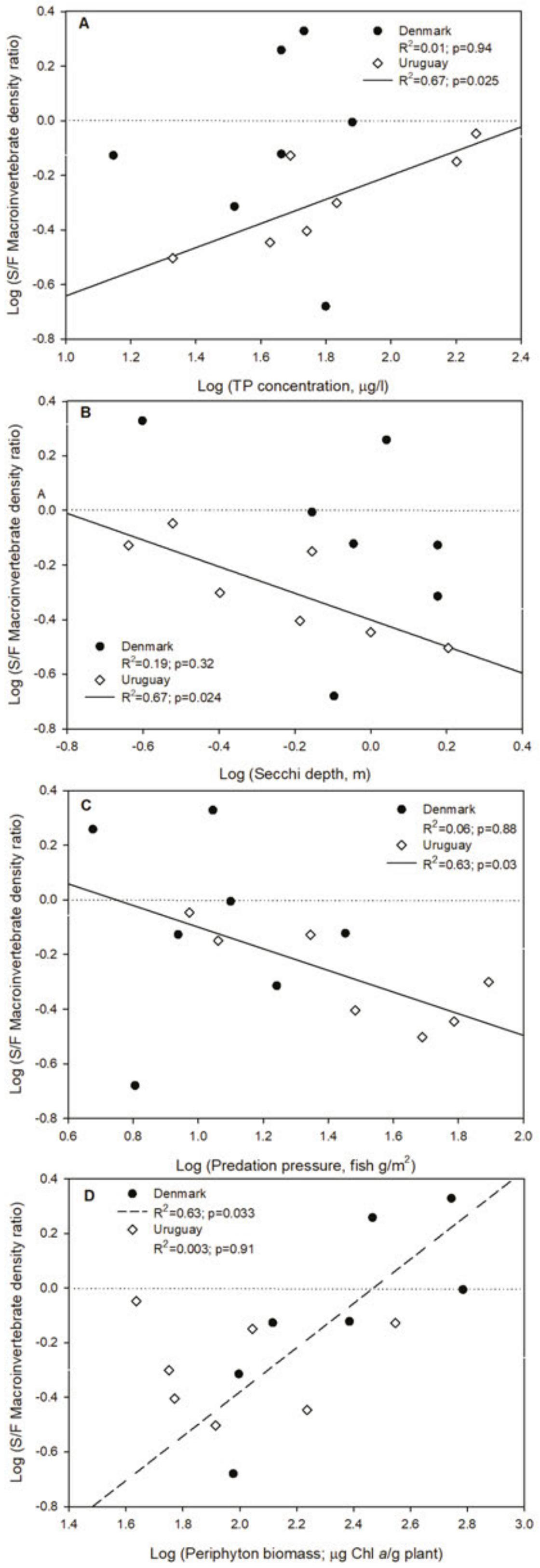

Figure 2. Use of artificial plant by macroinvertebrates (Submerged/Free-floating (S/F) density ratio) in the temperate (Denmark) and subtropical (Uruguay) lakes under different environmental conditions. From top to bottom: A. S/F density ratio and TP concentration, B. S/F density ratio and Secchi depth, C. S/F density ratio and potential predation pressure (fish biomass), and D. S/F density ratio and periphyton biomass. Variables were log10-transformed. All regression statistics are shown, but only the lines of significant regressions are plotted. The dotted line over 0 indicates equal use of free-floating and submerged plants; below 0 use of free-floating plants predominates and vice versa. *Uso de las plantas artificiales por los macroinvertebrados (cociente de la densidad entre plantas sumergidas y flotantes libres (S/F density ratio)) en los lagos templados (Dinamarca) y subtropicales (Uruguay), bajo distintas condiciones ambientales. De arriba abajo: A. Cociente S/F y concentración de PT, B. S Cociente S/F y profundidad de disco de Secchi, C. Cociente S/F y presión de depredación potencial (biomasa de peces), y D. Cociente S/F y biomasa de fitoplancton. Las variables fueron transformadas mediante log10. Se muestra el resultado de todas las regresiones, pero sólo de las significativas se muestra la línea de regresión. La línea punteada sobre el valor cero indica un uso igualitario entre los tipos de plantas, mientras que valores negativos indican una preferencia por las plantas flotantes libres y viceversa con las sumergidas.*

In the subtropical lakes, TP concentration and Secchi depth explained the variation in the S/F density ratio equally well (Fig. 2 A, B), but Secchi depth and fish biomass were selected for the final explanatory model (stepwise selection, $R^2 = 0.90$, $F_{2,4} = 18.16$, $p = 0.009$). As Secchi depth, fish biomass had a negative impact on the S/F density ratio (Fig. 2 C), causing larger differences in macroinvertebrate densities between the two plant types towards an even stronger use of free-floating plants with increasing transparency and an increasing potential predation pressure. In contrast, although macroinvertebrate densities were overall not significantly related to periphyton biomass in the temperate lakes, periphyton biomass significantly and positively explained the difference in abundance between plant types (expressed as the S/F density ratio) (Fig. 2 D).

DISCUSSION

We found distinctly different patterns in community structure, association to different habitat complexities, and relationships with environmental variables of littoral macroinvertebrates between temperate and subtropical regions.

Because we sampled only the macroinvertebrate assemblages associated with the artificial plants (i.e. littoral), we cannot rule out that other taxa could appear in plant-free or pelagic areas. However, based on works from other systems (e.g. Thomaz *et al.*, 2008; Boll *et al.*, 2012), we do not expect a climate bias in the potentially missing information. Besides, the observed pattern in macroinvertebrate richness was similar to that found for zooplankton in the same field experiment (Meerhoff *et al.*, 2007b), and plausible explanatory mechanisms emerge.

The major differences in the structure of the fish community in shallow lakes between the two climate zones have been identified as the main explanatory factor behind the observed differences in the structure of the potential prey communities (Meerhoff *et al.*, 2007a). Our results suggest that fish are more important than trophic state and local habitat complexity in structuring the richness, composition, and density of plant-associated macroinvertebrates. In both climate zones, amphipods were found in higher proportions in the lakes with the lowest fish biomass, most likely due to their high vulnerability to fish predation (Macneil *et al.*, 1999; Toft *et al.*, 2003). Also, Trichoptera and other insects, excluding Diptera, were found in relatively higher densities in the lakes with the lowest fish biomasses, indicating that these groups are vulnerable to fish predation too. The very narrow body size range of the subtropical chironomids in contrast to the relatively broad size range in the temperate lakes is also consistent with a stronger (and size-selective) predation by fish in the subtropics. In contrast, large-bodied shrimps, snails, and crabs were only found in the Uruguayan lakes and often occurred even at high fish biomass. Apple snails, shrimps, and crabs are large enough to escape predation at least by small fish, allowing for coexistence with the small fishes typically found in the littoral areas of subtropical lakes (Teixeira-de Mello *et al.*, 2009). An equivalent pattern has been recorded in brackish lakes in temperate areas where the shrimp *Neomysis integer* (Leach) coexists with the small sticklebacks *Pungitius pungitius* (L.) and *Gasterosteus aculeatus* (L.) (Jeppesen *et al.*, 1994).

In slight contrast to our results, an experimental study undertaken along a latitudinal gradient in shallow lakes in Europe (Miracle *et al.*, 2006) showed that fish affected the composition rather than the abundance of the plant-associated macroinvertebrate community and that the outcome of the fish-macroinvertebrate interaction depended markedly on the life history traits of the macroinvertebrates (Miracle *et al.*, 2006). However, the fish density in their study was relatively low and fixed in closed mesocosms, while fish were allowed to move freely between the open water and the artificial plants in our field experiment and in a similar experiment conducted by Brucet *et al.* (2012) in brackish shallow lakes in Denmark and Mediterranean Spain. The difference in the experimental design, with consequent differences in fish density and the potential predation pressure, therefore likely explains the different outcome of these experiments, and the broader impacts of fish detected in this study and in the Mediterranean shallow lakes. The lower macroinvertebrate richness and densities at high fish abundance in the subtropical lakes may result not only from the expectedly greater fish predation there (Teixeira-de Mello *et al.*, 2009; Meerhoff *et al.*, 2012), but also potentially from long-term strong competition with small omnivorous fishes (Flecker, 1992). Small subtropical fish might occupy trophic niches usually used by macroinvertebrates in other regions (Wootton & Oemke, 1992). Alternatively, as the biomass of periphyton was significantly higher on the artificial plants in the Danish lakes than in the Uruguayan lakes, this greater food availability could lead to a higher abundance of macroinvertebrates in the temperate lakes. However, the stronger role of predation in warmer climates is also evident from the comparative field study of Brucet *et al.* (2012) in brackish lakes, where drastically lower densities of macroinvertebrates occurred on artificial plants in Mediterranean lakes than in similar temperate lakes despite comparable periphyton biomasses.

Habitat complexity, however, interacts with fish characteristics. Rennie & Jackson (2005) found that small-scale variation in microhabitat complexity shaped the distribution of littoral macroinvertebrates, but they also found that such effects were systematically different relative to whether fish were present or absent. In their study, greater habitat complexity promoted higher density of macroinvertebrates only in the presence of fish, suggesting that habitat complexity effects are mostly mediated through top-down mechanisms. In a study of 104 shallow cold temperate lakes, fish assemblages explained > 11 % of the among-lake variance patterns in macroinvertebrates despite possible buffering effects by submerged plants (Hanson *et al.*, 2015).

Temperate macroinvertebrates often occur in higher abundance on submerged vegetation than on floating-leaved plants (Cremona *et al.*, 2008; van de Meutter *et al.*, 2008), arguably as a consequence of the higher structural complexity of the former. A comparative study found that the abundance of macroinvertebrates on natural submerged plants did not differ between simple and complex forms, whereas on artificial plants more invertebrates occurred on complex than on simple forms (Taniguchi *et al.*, 2003). In our study the abundance of macroinvertebrates on the artificial submerged macrophytes was almost always lower than on the free-floating macrophytes despite the higher % PVI of the submerged plant modules. However, variation in free or occupied space sizes has been highlighted as more important for macroinvertebrate taxonomic richness than overall complexity, plant biomass, and density of stems (St. Pierre & Kovalenko, 2014), and space size was indeed more variable under our artificial floating plant beds than on the more homogenously distributed submerged stems. Along the same line, Thomaz *et al.* (2008) recorded higher values of surface area available for colonization and fractal dimension (as a measure of complexity) in the root system of the anchored floating plant *Eichhornia azurea* (Sw.) Kunth than for the submerged *Egeria najas* Planch, and similar results were found for *E. crassipes* compared with the submerged *Cabomba furcata* Shult. & Shult.f. (Matsuda *et al.*, 2015), although such differences did not lead to differences in invertebrate densities.

The greater use of the free-floating plants by macroinvertebrates in the subtropics coincided with a lower use of these plants by the fish there (Teixeira-de Mello *et al.*, 2009). In most temperate lakes, littoral macroinvertebrates were more evenly distributed between the two plant types as shown by an S/F density ratio frequently closer to 1 than in the subtropical lakes despite the greater fish density in the free-floating plant modules (Teixeira-de Mello *et al.*, 2009). This could imply that the artificial free-floating plants acted as an even more effective refuge in temperate lakes or, most likely, that temperate fish prey less heavily on macroinvertebrates than their subtropical counterparts, thus allowing co-existence. The positive relationship between habitat use (S/F density ratio) and periphyton biomass in the temperate lakes also suggests that bottom up processes were more relevant there than in the subtropics where top-down processes seemed to rule.

Habitat complexity also interacts with water transparency (Snickars *et al.*, 2004; Pekcan-Hekim & Lappalainen, 2006), which can influence the fish predation pressure on macroinvertebrates by affecting visually hunting fish (Radke & Gaupisch, 2005; Pekcan-Hekim & Lappalainen, 2006). With increasing eutrophication, water turbidity might decrease the importance of plants as an anti-predation refuge. The refuge effect of the free-floating plants in our study was particularly observed under clear water conditions in the subtropical lakes. Perhaps as a consequence of a relatively lower need of refuge, the difference between plant types decreased with lower water transparency.

Free-floating plants are currently more common in warm lakes than in temperate regions, but, as a result of climate warming and increased eutrophication, the geographical distribution of free-floating plants is likely to expand (Scheffer *et al.*, 2003). Fish predation pressure is also expected to augment with climate warming (Meerhoff *et al.*, 2012). Even though we found that patches of free-floating macrophytes may be a comparatively better refuge (or preferred habitat) for macroinvertebrates than submerged plants, free-floating plant dominance in lakes often leads to anoxia and biodiversity loss. Maintenance of a diverse macrophyte community in shallow lakes thus seems important to facilitate coexistence between plant-associated macroinvertebrates and fish and to allow linkage between littoral, benthic, and pelagic habitats.

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Palaeolimnology

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Dry and wet periods over the last millennium in central-eastern Spain - a paleolimnological perspective

Charo López-Blanco[1,*] and Lidia Romero-Viana

[1] Animal Ecology and Systematics. Justus Liebig University Giessen, Heinrich-Buff-Ring 26 (IFZ) D-35392, Giessen (Germany).

[*] Corresponding author: Charo.Lopez-Blanco@allzool.bio.uni-giessen.de

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ABSTRACT

Dry and wet periods over the last millennium in central-eastern Spain-a paleolimnological perspective

A compilation of sedimentological, chemical and mainly biological proxies from sedimentary records in three lacustrine systems (La Cruz, El Lagunillo del Tejo and El Tobar) in the Iberian Range provides a synthetic view of hydroclimatic variability in central-eastern Spain during the last millennium. A quantitative rainfall reconstruction from varved sediments in Lake La Cruz was used to calibrate the sedimentary inputs of five biological proxies (photosynthetic pigments, diatoms, cladoceran sub-fossils, plant macrofossils and stable isotopes in authigenic carbonates) in the longer sequence of the nearby Lagunillo del Tejo. In spite of the resolution and the distinct responses of the proxies analysed, the three reconstructions are internally coherent and consistent with available North Atlantic Oscillation reconstructions. The Medieval Climate Anomaly (MCA; AD 900-1300) was marked by changes in humidity but it was generally wet, which contrasts with some reconstructions for Iberia and offers interesting information about the spatial heterogeneity of this period in the region. A drier period (AD 1300-1400) occurred at the onset of the Little Ice Age (LIA; AD 1300-1850), which was generally marked by increasing precipitation. The 20th century generally records higher humidity except from the beginning of the century. The recovery of longer and higher resolution lake sediments in this key region offers exceptional scientific opportunities for climate research.

Key words: Hydroclimate reconstruction, lake sediments, climate proxies, North Atlantic Oscillation, last millennium

RESUMEN

Periodos secos y húmedos durante el último milenio en el centro-este de España- una perspectiva paleolimnológica

En este estudio se ofrece una recopilación de indicadores sedimentarios, químicos y especialmente biológicos de testigos sedimentarios de tres sistemas lacustres (La Cruz, El Lagunillo del Tejo y El Tobar) en el Sistema Ibérico, que proporciona una visión sintética de la variabilidad hidroclimática en el centro-este de España durante el último milenio. Se utilizó la reconstrucción cuantitativa de la lluvia a partir de los sedimentos varvados del lago La Cruz para calibrar la señal sedimentaria de cinco indicadores biológicos (pigmentos fotosintéticos, diatomeas, sub-fósiles de cladóceros, macrofósiles de plantas e isótopos estables en carbonatos autigénicos) en una secuencia más larga procedente del Lagunillo del Tejo. A pesar de las distintas resoluciones y respuestas de los indicadores analizados, las tres reconstrucciones muestran concordancia entre ellas y son consistentes con la Oscilación del Atlántico Norte (NAO). La Anomalía Climática Medieval (AD 900-1300) estuvo marcada por cambios en la precipitación pero fue un periodo generalmente húmedo, lo cual contrasta con algunas reconstrucciones en la Península Ibérica y ofrece información interesante sobre la heterogeneidad climática de este periodo en la región. Entre AD 1300 y 1400 se registraron valores bajos aunque la Pequeña Edad del Hielo (AD 1300-1850) estuvo marcada por un progresivo aumento de la precipitación. El siglo XX registró niveles de precipitación mayores, excepto a principios de siglo. Secuencias más largas y con una resolución más alta en esta región ofrecerían excepcionales oportunidades científicas para la investigación climática.

Palabras clave: *Reconstrucción hidroclimática, sedimento lacustre, indicadores climáticos, Oscilación Atlántico Norte, último milenio*

INTRODUCTION

Climate change projections for the Iberian Peninsula (IP) suggest both an increase of temperature (by 1.5 ºC to 3.6 ºC in the 2050s) and also precipitation decreases in most of the territory, which may indicate an increased likelihood of droughts (Iglesias, 2009). According to several studies, these effects have already started in the IP (Vicente-Serrano & Cuadrat-Prats 2006; García-Herrera *et al.*, 2007; González-Hidalgo *et al.*, 2009). Iglesias *et al.* (2007) shows that drought events, defined as a natural episodes that exists when precipitation is significantly below normal recorded levels (UN Secretariat General, 1994), have been more frequent since AD 1970 in Spain. Documented data series on precipitation in the IP are scarce, and those that exist are relatively short. The longest data series, for San Fernando (Cadiz), records precipitation only from AD 1854. Natural archives such as tree rings, ice cores, stalagmites and lake sediments can be used to extend instrumental data back in time and thus, to gain a wider perspective on climatic variables.

Many hydroclimatic reconstructions using lake sediments from IP have been published in the last decade (e.g. Moreno *et al.*, 2008-Lake Taravilla; Morellón *et al.*, 2011-Lake Estanya; Corella *et al.*, 2014-Lake Montcortés; Barreiro-Lostres *et al.*, 2014-Lake La Parra). These studies have mainly focused on sediments from the last millennium, which include key periods for understanding hydroclimatic variability such as the Medieval Climatic Anomaly (MCA; ca. AD 900-1300) and the Little Ice Age (LIA; ca. AD 1300-1850). Some sedimentary sequences also extend beyond this period, and even as far as the onset of the Holocene (e.g. Martín-Puertas *et al.*, 2010; Corella *et al.*, 2011; Morellón *et al.*, 2018; Moreno *et al.*, 2012; Sánchez-López *et al.*, 2016). Unlike most of these studies, which have mainly used sedimentological and geochemical approaches to infer climate variability, Rosa Miracle´s research group at the University of Valencia worked with biological proxies in the Cañada del Hoyo lakes (Cuenca Mountains, Iberian Range) and in L'Albufera, a coastal lagoon in Valencia (eastern Spain). These systems were very well-known to the research group, who had started studying their limnological features at the beginning of the 1980s (Miracle & Vicente,1983; Garcia *et al.*,1984; Serra *et al.*, 1984; Miracle, 1984; Oltra & Miracle, 1984; Vicente & Miracle, 1984). In the mid-2000s, the research line in paleolimnology lead by Rosa Miracle was one of the most active research groups in Spain, as shown by the group´s key publications (i.e. Romero-Viana *et al.*, 2006; 2008; 2009 a, b; 2010; 2011; López-Blanco *et al.*, 2011; 2012a, b; 2013a, b; 2016 a, b; Marco-Barba *et al.*, 2013a, b). Rosa Miracle was an exceptional scientist with a global ecological perspective. She contributed to building the bridge between neo- and paleolimnology, improving lake sediment interpretations and consequently increasing our knowledge of past climatic variability (SIL news, 2017).

Fifteen years after the first paleolimnological studies by Rosa Miracle's research team, we publish a review of three lacustrine sedimentary sequences (La Cruz, El Lagunillo del Tejo and El Tobar). This is our tribute to our mentor and thesis director, who was one of the most important limnologists of recent years. This paper offers a synthetic view of hydroclimate in central-eastern Spain, which will contribute to gaining a wider overview of the hydroclimatic situation in this key area.

LACUSTRINE SYSTEMS AND SEDIMENTARY SEQUENCES

The sinkholes

The three lacustrine systems studied, La Cruz, El Lagunillo del Tejo and El Tobar are dolines located in the southern part of Iberian Ranges at an altitude of ca. 1000 m a.s.l. (Fig. 1). The study area is characterised by a Mediterranean climate with a typical seasonal pattern of very dry, hot summers and cooler, rainier winters (Csa type according to the climatic characterisation of Köppen, Atlas climático de España, 2011) (Fig. 2). The total annual rainfall is 525 ± 123 mm (mean of instrumental data series 1950–2003 from the nearby town of Cuenca). Regional winter precipitation, contributing 50 % of the total amount, is highly correlated with the phase of the North Atlantic Oscillation (NAO) (Rome-

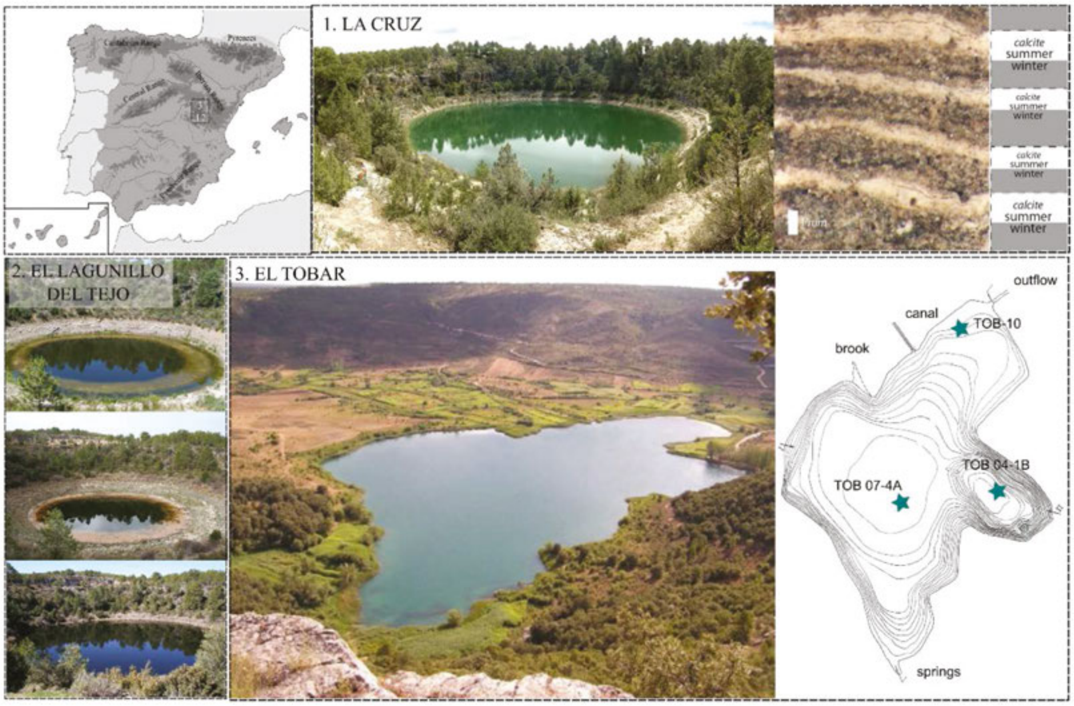

Figure 1. Situation of the lakes in the IP and photographs of each system. Note that photographs from Lagunillo del Tejo show lake level changes in July 2007, March 2008 and April 2010 from top to bottom. In the upper right corner micrography of La Cruz laminated sediment. *Situación de los lagos lagos en la PI y fotografías de cada sistema. Las fotografías del Lagunillo del Tejo muestran los cambios de nivel en julio 2017, marzo 2008 y abril 2010 de arriba a abajo. En la esquina superior derecha micrografía del sedimento laminado en La Cruz.*

ro-Viana *et al.*, 2008; López-Blanco *et al.*, 2016a) (Fig 2). The selected lakes have two common features: 1) a karstic origin and 2) a carbonate (or evaporate) bedrock.

Lake La Cruz and Lagunillo del Tejo are in close proximity. They belong to the karstic complex of Cañada del Hoyo, which is composed of 34 sinkholes and the polje crossed by the Guadazaon river. They were developed in Cretaceous dolostones (Cenomanian-Turonian stages) through dissolution and fracture processes in the Pliocene (Gutiérrez-Elorza & Pena-Monne, 1998). The lateral expansion of the Valdemoro fault and the NW-SE anticlinal folding (Eraso, 1979) may favour water capture with seven sinkholes intercepting the phreatic level (Escudero & Regato, 1992). La Cruz is significantly bigger than the Lagunillo del Tejo (Fig. 1). The first has a circular surface with a mean diameter of 122 m and a maximum depth of 21 m. Water fills the bottom of a sinkhole of greater dimensions (170 m mean diameter with walls standing 16-25 m above the water level). Lake La Cruz is currently meromictic with a permanently anoxic monimolimnion below 18 m. A complete review of La Cruz features has been published recently in Camacho *et al.* (2017). One peculiarity of this lake is the annual summer whiting through a short-term massive $CaCO_3$ precipitation process (Rodrigo *et al.*, 1993; Miracle *et al.*, 2000). The water turns suddenly turbid and just few days after surface becomes clearer, with whiting ending in one or two weeks. Although temperature could control the abrupt precipitation, the high number of picocyanobacterial cells collected by the sediment traps after whiting (Camacho *et al.*, 2003) suggest that biogenic processes might be also involved in the precipitation. Seasonal pulses of calcite crystal precipitation are responsible for varve sediment formation (Fig. 1).

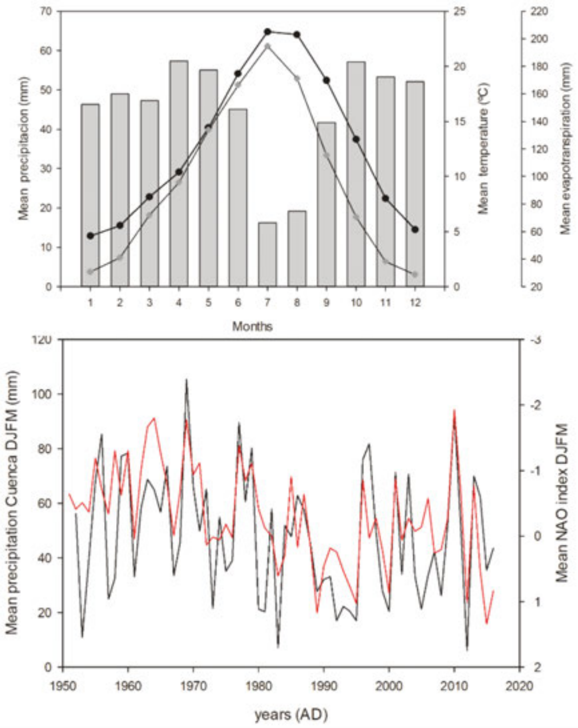

Figure 2. Regional climate conditions. A) Monthly precipitation and mean temperature and B) Correlation between winter rainfall and NAO index (r = -0.66, *p* < 0.01). *Condiciones climáticas regionales. A) Precipitación mensual y temperatura media y B) Correlación entre la precipitación de invierno y el índice NAO (r = -0.66,* p *< 0.01).*

Lagunillo del Tejo is also fed by groundwater and is subjected to marked water-level fluctuations (Vicente & Miracle, 1984; Romero-Viana *et al.*, 2009b). The lake is monomictic, thermally stratified from May to early autumn. An anoxic and deep hypolimnion develops in summer only during high-water level. Conversely, the whole water column remains mixed during periods of low water level (< 5 m) (Vicente & Miracle, 1984). Due to its particular shape, changes in lake level drastically affect the lake diameter (see Fig.1).

From a limnological and morphological point of view, Lake El Tobar is quite different from La Cruz and El Lagunillo. It is also a karstic lake situated northern of the Canada del Hoyo complex, in Jurassic and Cretaceous dolostones, but it is much larger, open and it has two different sub-basins (Fig.1). The holomictic sub-basin is larger, elongated and has a maximum depth of 12.8 m. The meromictic sub-basin is smaller but deeper, circular and has a maximum depth of 19.5 m (Vicente *et al.*, 1993). The lake basin was formed by solution of carbonated rocks and the underlying evaporitic Keuper formations. The latter is evident from the meromictic sinkhole, which has a saline water layer below 12 m that is permanently anoxic (Vicente *et al.*, 1993). The lake is naturally fed mainly by freshwater from subaquatic springs on the Eastern shore and, to a much lesser extent, by a surface water brook. There is also a canal, which was built between AD 1964 and AD 1966 to transfer water from Reservoir La Tosca to Lake El Tobar. El Tobar has functioned as a regulatory water reservoir since this connection (López-Blanco *et al.*, 2011).

The exceptional varved sediment of La Cruz

The most outstanding feature of the La Cruz sediment is an excellently preserved laminated structure in the uppermost 40 cm (Julià *et al.*, 1998) (Fig. 1). This is formed by couplets of alternated white and brownish laminations. The light laminae are composed of calcium carbonate crystals deposited after the summer whiting event each year. The dark laminae consists of organic silts with a minor section of fine mineral clasts (Romero-Viana *et al.*, 2008). Each couplet, called a varve, therefore represents an annual cycle of sedimentation.

A calibration study confirmed the potential use of laminations as a quantitative climate proxy (Romero-Viana *et al.*, 2008). In this study, the rainfall and temperature data series recorded in the region from AD 1950 onwards were compared with lamination thickness. The results have shown that accumulated winter rainfall, from December to March, is the best predictor of calcite lamina thickness. A monitoring study carried out in Lake La Cruz over three consecutive years (AD 1996-1998), considering sediment traps and the water column data available supported this relationship (Miracle *et al.*, 2000; Romero *et al.*, 2006). Although high water temperature and photosynthetic activity (Camacho *et al.*, 2003) are trigger factors of the massive calcite precipitation during summer, the total amount of crystal precipitated in the lake during summer whiting depends on annual dissolved calcium renewal, which in turn is dependent on the aquifer discharge after winter rainfall (Miracle *et al.*, 2000; Romero-Viana *et al.*, 2008).

Given the relationship between rainfall and calcite lamina thickness, Romero-Viana *et al.* (2011) attempted the reconstruction of annual rainfall from December to March over the last centuries. Three sediment cores were used to construct the varve chronology. The sediments were freeze-dried in the laboratory and hardened with epoxy resin. Thin sections were obtained and scanned to obtain high-resolution digital images. The number and thickness of laminae present in each thin section were determined between visually discernible marked horizons using the measurement tools in image analyses software UTHSCSA (ftp://maxrad6.uthscsa.edu). The three sequences were cross-matched according to sedimentological criteria the occurrence of detrital layers and the number of varves between detrital events. After the sequences cross-matching, a total of 423 annual laminations were then identified; confirming the onset of laminated sediments from AD 1579. Annual laminations provide better chronological data than radioisotopic methods, however, lamination chronology has been tested by an independent dating method. The chronological model based on ^{210}Pb activity (Romero-Viana *et al.*, 2009a) matches the varve dating.

To identify the climate signal, each raw calcite laminae thickness series was normalised, resulting in three dimensionless annual index series. The three index series were averaged year by year to produce a master series increasing the climate signal and partially cancelling non-climatic noise. Annual winter (DJFM) rainfall values were inferred using the calibration function described in Romero-Viana *et al.*, (2008) after adding new data corresponding to the series CV-98 (AD 1950-1988). The accuracy of rainfall

Table 1. Compilation of the sediment cores recovered in Lagunillo del Tejo. The table compiles information about the core names, year of recovery and place of collection, recovery methods, analysed proxies in each core and the articles published by the group in this system. *Recopilación de los testigos sedimentarios recuperados en el Lagunillo del Tejo. La tabla muestra información sobre los nombres de los testigos, el año y lugar de muestreo, el método de recuperación, los indicadores analizados y los artículos publicados por el grupo en este sistema.*

Core name	Year	Location	Recovery method	Proxy	References
CN1	2003	Center	Livingston core	Photosynthetic pigments and diatoms	Romero-Viana *et al.* (2009b, 2009c)
CN2	2005	Center	Livingston core	Dating	Romero-Viana *et al.* (2009b, 2009c)
CN3a	2008	Center	Russian peat corer	Cladocerans	Romero-Viana *et al.* (2009a); López-Blanco *et al.* (2012b); López-Blanco *et al.* (2013b)
CN3b	2008	Center	Russian peat corer	Dating	López-Blanco *et al.* (2011, 2012)
CN4	2009	Littoral	UWITEC gravity corer	Macrofossils, charcoals and stable isotopes	López-Blanco *et al.* (2012a,b); López-Blanco *et al.* (2016a)

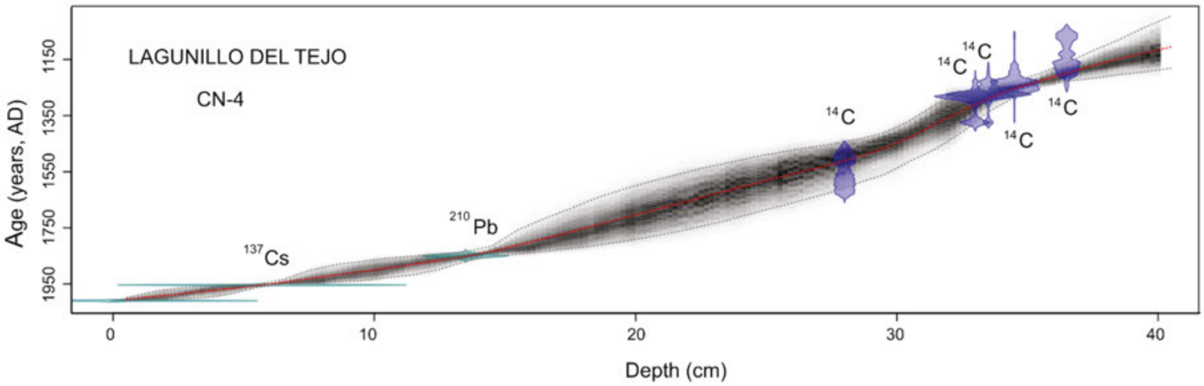

Figure 3. Chronological model in El Lagunillo del Tejo based on weighted spline regression (Blaauw, 2010) of five AMS ^{14}C dates, ^{210}Pb and ^{137}Cs dates. *Modelo cronológico del Lagunillo del Tejo basado en regresión lineal ponderada (Blaauw, 2010) de cinco dataciones de AMS ^{14}C dates y dataciones superficiales de ^{210}Pb and ^{137}Cs.*

reconstruction was verified by comparing the reconstructed values for AD 1859 to AD 1949 with the available instrumental data series from Cuenca and Madrid (Romero-Viana *et al.*, 2011). The positive value of the reduction error (RE) measure and the coefficient of efficiency (CE) confirmed the skill of this reconstruction.

The smallest lake and the longest record: El Lagunillo del Tejo

From 2003 to 2009, five sediment cores were recovered from Lagunillo del Tejo in order to study photosynthetic pigments, diatoms, cladocerans, plant macrofossils, charcoals and stable isotopes. The presence of conspicuous oxidised and reduced layers in the lithology together with a distinctive pattern in physical properties (density, water content, organic matter and carbonates) in all the analysed sediment cores, allowed correlations to be identified. Table 1 shows the complete list of sediment cores, locations, recovery methods, studied proxies and references.

A preliminary chronological model applying the constant initial concentration method (CIC) was established according to the activity of two different radionuclides (^{137}Cs and ^{210}Pb) (Romero-Viana *et al.*, 2009a, b). Afterwards, terrestrial plants and charcoal macroremains from CN-3 and CN-4 cores were then used for accelerator mass spectrometry (AMS). An age-depth model was established on the basis of the ^{210}Pb and ^{137}Cs dates and five AMS dates though an interpolation of the calibrated ages. This chronological model was published in López-Blanco *et al.* (2012) and was remodelled for this paper using the new tools for Bayesian statistical age-models in the Bacon package (Blaauw & Christen, 2011) in R software. This new model includes ^{137}Cs peak, ^{210}Pb model and the five available ^{14}C dates (Fig. 3). The radiocarbon dates were calibrated using the IntCal13 calibration curve (Reimer *et al.*, 2013) and the 95.4 % distribution (2δ probability interval) was taken into account to build the age-depth model. The results were very similar to those obtained in the AD 2012 model but more accurate since the Bayesian approach considered the chronological dates in the stratigraphy to build the model in terms of probability. The chronological uncertainties published by López-Blanco *et al.* (2012a) in the middle of the sequence were partially solved by this approach (Fig. 4). New extrapolations beyond dated samples at the bottom of the core, and taking into account the calibrated ages and the 2δ distribution in the new model, yield an age of AD 1112 for the bottom of the core (40.5 cm). López-Blanco *et al.* (2012a) estimated the age of this core as around AD ca. 1100 with accumulation rates of 0.26 mm/ yr between AD 1100 and AD 1530, 0.44 mm/yr to AD 1850 and 0.87 mm/y in the top layers. This new chronological model completes and confirms the previous age-depth relationship in CN-4. However, there are still uncertainties when running the chronological model in the longest core (CN-3; 72.5 cm) since the latest dating was done at 47.5 cm from this sediment core (López-Blanco *et al.*, 2012a). The extrapolation of dates beyond the last dated level results in a wider range of possible ages for certain depths at the bottom of CN-3 sediment core (from 47.5 to 72.5 cm).

Up to 60 different photosynthetic pigments were identified in the Lagunillo del Tejo sediment core (Romero-Viana *et al.*, 2009b). In addition to chlorophyll derivatives, specific carotenoids such as zeaxanthin, alloxanthin and also bacteriochlorophylls a and d were used as tracers of algal and bacterial populations, respectively. Given the high number of variables, it was necessary to apply a principal component analysis (PCA). PCA of specific pigment depth profiles suggested two different ecological communities that have switched in relative importance during the past centuries in response to lake-level variability; 1) (positive values PC) a planktonic group of algal populations comprising chlorophytes, cryptophytes, cyanobacteria, and phototrophic bacteria populations associated with higher lake level and water column temporal stratification; and 2) (negative values, PC) a littoral community with benthic and epiphytic alga and macrophytes, indicators of lower level.

López-Blanco *et al.* (2013) showed that sediment from the central part of this small lake truly represent the whole cladoceran community, which means that the relative abundance of cladocerans was a good descriptor of the overall community in the lake at one determining point of time. Cladoceran assemblages were composed of one planktonic (*Daphnia longispina* group) and 16 littoral species. The crustacean community was typical from shallow karstic sinkholes in the Serranía de Cuenca. The whole sedimentary sequence was characterized by an alternation of planktonic and littoral cladocerans. Within the littoral cladocerans, there was also an alternation between cladocerans highly associated with macrophytes (i.e. *Graptoleberis testudinaria*) and facultative planktonic cladocerans (i.e. *Chydorus sphaericus*). The planktonic/benthic ratio (P/B ratio) was not a good proxy of lake level changes in this ecosystem due to the shape, littoral configuration and distribution of cladoceran populations according to habitat preferences. López-Blanco *et al.* (2012b) showed that the main factor controlling the cladoceran community in this lake was the configuration of the littoral zone, which depends on the lake water depth. At higher lake levels, the lake had two rings of macrophytes and the sedimentary signal was comprised of a higher relative abundance of phytopilous cladocerans. When water level reduced, the outer ring of macrophytes (*Chara* spp, *Ranunculus* subsg Batrachium, and *Nitella*) dried out together with all the associated cladoceran community. The sedimentary signal in such circumstances was composed of a high relative abundance of *Daphnia longispina* group. This early hypothesis was confirmed by analysing plant macrofossils record in the sediment, as shown in López-Blanco *et al.* (2012a) and López-Blanco *et al.* (2012b).

Although diatom concentration in Lagunillo del Tejo was low, 34 diatom taxa (26 genera) were identified (Romero-Viana *et al.*, 2009c). Diatom assemblages were mainly dominated by peri-epiphytic genera, and characterized by mesotrophic and alkaliphylic taxa. The increase of tycoplanktonic diatoms was associated to lake level drops.

Isotopic ratios of C and O from authigenic carbonates were used in Lagunillo del Tejo to extract important information about the basin hydrology, and to confirm all the inferences about past lake level changes inferred from biological proxies. Analysis of modern water from the lake plot below the Global Meteoric Water Line, indicated that evaporative processes control the lake´s isotopic composition. Sediment samples showed a covariant trend between carbonate $\delta^{18}O$ and $\delta^{13}C$, showing that the precipitation/evaporation ratio largely controlled the isotopic composition of this lake and was used to reconstruct the hydrological past of this system. Higher isotopic ratios were interpreted as arid periods while low isotopic ratios corresponded to wetter periods. Evaporation is higher during arid conditions, and authigenic lake carbonates are enriched in ^{18}O. During wetter periods the situation reverses, and the $\delta^{18}O$ of the lake water reflects the

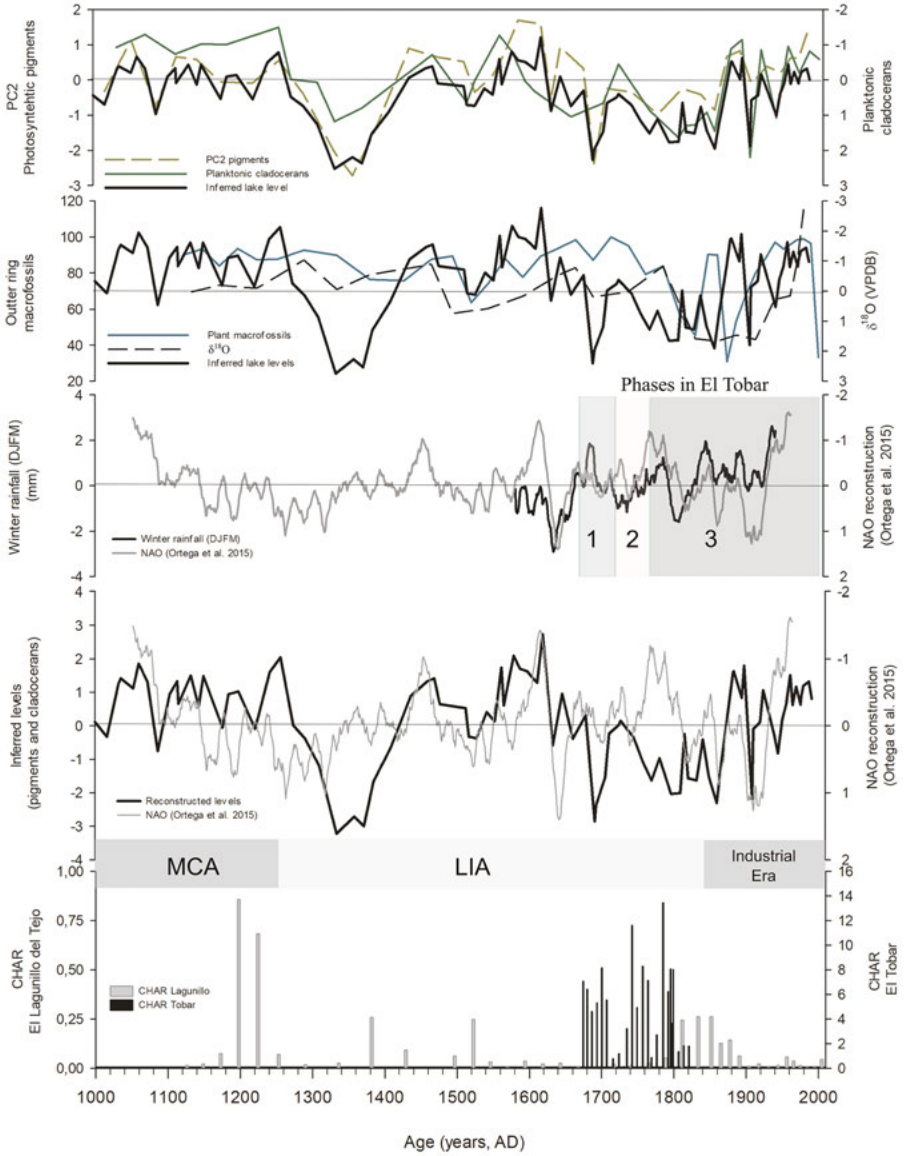

Figure 4. Compilation of the results obtained in the three lakes. From upper to bottom panels. 1) PC2 from photosynthetic pigments, normalized data of planktonic cladocerans (Romero-Viana *et al.*, 2007), and reconstructed levels based on mean values of these variables; 2) Percentages of plant macrofossils from the outer ring (López-Blanco *et al.*, 2012a), normalized date of δ^{18}O (López-Blanco *et al.*, 2016a) and reconstructed levels based on pigments and cladocerans; 3) Comparison of the Winter Rainfall signal inferred in La Cruz (Romero-Viana *et al.*, 2011) , the NAO reconstruction (Ortega *et al.*, 2015) and phases of transgression and regressions in the shore line of Lake El Tobar (López-Blanco *et al.*, 2016b); 4) Comparison of the inferred lake level in El Lagunillo with the NAO reconstruction (Ortega *et al.*, 2015); 5) Charcoal signal (in charred particles/cm^3.yr) in El Lagunillo and El Tobar. Note that all the normalization was done by using the mean and standard deviation. *Recopilación de los resultados obtenidos en los tres lagos. De arriba abajo: 1) PC2 de pigmentos fotosintéticos, datos normalizados de cladóceros planctónicos (Romero–Viana* et al.*, 2007), y reconstrucción de los niveles basado en valores medios de estas variables; 2) Porcentajes de macrofósiles de plantas del anillo exterior (López –Blanco* et al.*, 2012a), datos normalizados de* δ^{18}*O (López-Blanco* et al.*, 2016a) y niveles reconstruidos basados en los pigmentos y cladóceros; 3) Comparación de la señal de lluvia de invierno inferida en La Cruz (Romero-Viana* et al.*, 2011), la reconstrucción de la NAO (Ortega* et al.*, 2015) y las fases de trasgresión y regresión del litoral de El Tobar (López-Blanco* et al.*, 2016b);4) Comparación de los niveles inferidos en El Lagunillo del Tejo con la reconstrucción de la NAO (Ortega* et al.*, 2015); 5) Señal de carbones (en partículas quemadas/cm*^{3}*.yr) en El Lagunillo y El Tobar. Todas las normalizaciones han sido hechas utilizando la media y desviación estándar.*

lighter isotopic composition of the recharge (López-Blanco *et al.*, 2016a).

Past changes in the El Tobar shore line

Due to the complex morphological characteristics of Lake El Tobar, sediment cores were recovered from both deeper-meromictic and shallower-holomictic sub-basins to better represent the diverse communities inhabiting the lake (Fig. 1; Table 2).

The radioisotopic activity of ^{210}Pb and ^{137}Cs published in López-Blanco *et al.* (2011) and López-Blanco *et al.* (2016b) shows very different sedimentation rate in the central and littoral parts of lake El Tobar. TOB04-1B and TOB07-4A only covered the previous 60 years while TOB-10 dated back the last 350 years. Therefore, the central core recorded the eutrophication process and species introduction in the lake after canal construction (López-Blanco *et al.*, 2011).

The overlapping sedimentary sequence using the three consecutive littoral cores (TOB10) was characterised by the presence of three sedimentary units and five facies; deeper water facies were located in the uppermost part of the sequence (59-0 cm) while shallower-water facies were in the bottom part of the core (120-60 cm) (Fig. 5). Plant macrofossils from 18 taxa were identified in the same sedimentary sequence. Based on their occurrence and ecological characteristics (aquatic, marsh or terrestrial environment), five different periods of changes in shore line were found. The isotopic signal complemented and supported this information with data about present and past lake hydrology (López-Blanco *et al.*, 2016). Modern surface water samples for hydrogen and oxygen isotope analyses indicated that precipitation was the main factor governing the isotopic composition of lake water. The δ^{13}C profile of bulk carbonates showed lower values (around 5 ‰) at the bottom of the core and less negative

Table 2. Compilation of the sediment cores recovered in El Tobar. The table compiles information about the core names, year and place of collection, the recovery methods, the analysed proxies in each one and the articles published by the group in this system. *Recopilación de los testigos sedimentarios recuperados en el El Tobar. La tabla muestra información sobre los nombres de los testigos, el ano y lugar de muestreo, el método de recuperación, los indicadores analizados y los artículos publicados por el grupo en este sistema.*

Core name	Year	Location	Recovery method	Proxies	References
TOB04-1B	2004	Meromictic sub-basin	Livingston	Chronology Lithology Cladocerans Pigments and diatoms	López- Blanco *et al.* (2011)
TOB07-4A	2007	Holomictic sub-basin	Livingston	Chronology Lithology Cladocerans Pigments and diatoms	López-Blanco *et al.* (2011)
TOB10-1 TOB10-2 TOB10-3 (Composite core TOB10)	2010	Holomictic	Russian peat corer	Plant macrofossils Isotopes Charcoal analysis	López-Blanco *et al.* (2016b)

values in the two upper-thirds of the sediment record (Fig. 5). These changes were also evident in the $\delta^{18}O$ profile, with the lowest values at the bottom and highest in the middle. Additionally, several peaks in the $\delta^{18}O$ profile were detected in the middle part of the sediment core. The most plausible explanation for the $\delta^{18}O$ peaks observed in the El Tobar sequence was a change in drainage characteristics and residence time in littoral environments resulting in varying degrees of evaporative enrichment, with all of these processes occurring during the LIA due to changes in precipitation.

REGIONAL HYDROCLIMATIC SIGNAL

The sediments from La Cruz, El Lagunillo del Tejo and Lake El Tobar provide evidence for climatic changes during the last centuries in the Iberian Range. Unlike La Cruz and El Tobar sequences, the sediment cores from El Lagunillo del Tejo fortunately cover the whole last millennium. Hydroclimatic variability in this lake is recorded as successive lake level changes, which determine: i) the sedimentary inputs of photosynthetic pigments, ii) diatom, cladoceran subfossils and plant macrofossils signal and iii) stable isotope ratios in endogenic carbonates (Table 1, Fig. 4).

The Lagunillo del Tejo sedimentary sequence as a whole shows good agreement between all the indicators for the major arid and humid phases and reinforces the robustness of past lake level reconstruction (Fig. 4). Small differences between the proxies are partially related to the correlation methodology used between sediment cores and to the level of sample resolution. Correlation was

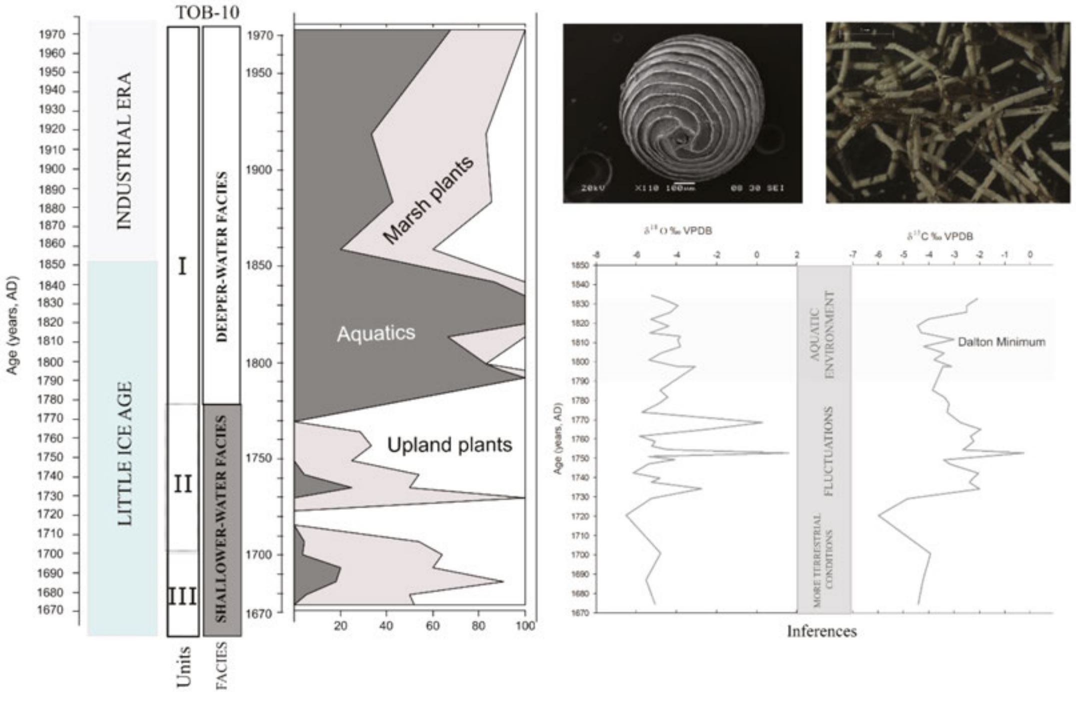

Figure 5. Compilation of the results obtained in littoral cores (TOB10-1, TOB10-2 and TOB10-3) from El Tobar. In the figure, only the composite TOB-10 is represented for sake of simplicity. From left to right: Main climatic periods, sedimentary facies and units, relative abundance of aquatics, marsh and upland plants and $\delta^{18}O$, $\delta^{13}C$ signals. Pictures in the upper right part show a gyrogonite and calcifications from *Chara* sp., which were quite abundant during the aquatic phase. *Recopilación de los resultados obtenidos en los testigos litorales (TO10-1, TOB10-2 y TOB10-3) de El Tobar. En la figura, solo se representa la secuencia solapada TOB-10 por simplificación. De izquierda a derecha: periodos climáticos principales, facies y unidades sedimentarias, abundancias relativas de plantas acuáticas, de marjal y terrestres y señales de $\delta^{18}O$, $\delta^{13}C$. Las fotografías en la parte superior derecha muestran un girogonito y las calcificaciones de* Chara *sp., que eran muy abundantes durante la fase acuática.*

based on physical properties such as density or organic matter, and although the profiles fitting was precise, this factor cannot be ruled out as responsible for small differences. The proxy signal for photosynthethic pigments (core CN-1, Table 1) also corresponded to discrete values of about 10, 5 and 3 years due to the different sedimentation rate throughout the sediment core with a temporal resolution of ca. 30 years. The age resolution in the case of cladocerans (CN-3, Table 1) is about 15 years in the upper part of the sequence and 35 years at the bottom, and the resolution of plant macrofossils and isotopes is ca. 10, 25 and 40 years in few samples at the bottom. Other factors, such as the different climatic sensitivities of the proxies, can also explain the discrepancies found in some specific periods.

According to inferred lake levels in El Lagunillo del Tejo, the MCA (ca. AD 900-1300) was a period marked by rainfall oscillations but generally wet. This agrees with records from north and/or western IP, which indicate an increase in humidity during the MCA (references in Moreno *et al.*, 2012). Despite this, many lacustrine records from the Mediterranean IP such as Basa de la Mora, Estanya and Arreo in the northeast and Zoñar in the south seem to indicate drier conditions (Moreno *et al.*, 2012). Notably our results showed a very good agreement with the lake level fluctuations inferred in the nearby Lake La Parra (Barreiro-Lostres *et al.*, 2014) (Fig. 6 confirming relatively wetter conditions during MCA in the Iberian Ranges. Although the onset of the LIA was not equally registered by all our proxies, they all agree with the idea of a progressive increase of level from the onset of the LIA to ca. AD 1550. Relatively humid conditions during the fifteenth and sixteenth centuries were directly related to the onset of the meromixis in La Cruz (Julià *et al.*, 1996) and coincided with the beginning of calcite lamination deposition in AD 1579 (Romero-Viana *et al.*, 2011). In this period, the El Lagunillo del Tejo lake level reconstruction overlaps with the La Cruz signal, which serves as a chronological and quantitative anchor for hydroclimate calibration. After the Maunder Minimum (AD 1645-1715), there may have been an increase in humidity according to the La Cruz record, the plant macrofossils record of El Lagunillo and all the indicators in El Tobar. However, the three sequences also show continuous changes in hydrovariability through the whole seventeenth century, with a dry period ca. AD 1690. Barreiro-Lostres *et al.* (2014) also inferred a highly variable but humid period from the occurrence of sandy layers intercalated within fine-laminated facies in the nearby Lake La Parra. The isotopic signal and sedimentology from El Tobar strongly support the idea of an alternation of humid and drier periods that could have altered the lake recharge during the changing precipitation conditions in the late LIA. Benito *et al.* (2003) described five periods of concentrated floods events in the Tagus drainage basin in: AD1200-1230; AD 1560-1620; AD 1700-1720; AD1740-1810; AD 1860-2000, which agrees well with the transgression and regressions of the shore line described in El Tobar, which is located in Tagus headwaters, but also with the hydrological reconstruction in El Lagunillo for the whole millennium. At a more local scale, inferences from the nearby Taravilla Lake (Moreno *et al.*, 2008), together with unpublished results from Benito about historical floods from the Guadiela River (Fig. 6) also support the increase in level inferred from phase 2 to 3 in El Tobar. Higher sediment delivery (events S2, S3 and S4) (Fig. 6) inferred from a distal core in El Tobar could be the results of synergic effects of humid periods and human impacts during the 19 and 20th centuries (Barreiro-Lostres *et al.*, 2014). In spite of the differences in size, hydrology, limnology and sensibility to rainfall oscillations, the three main phases of lake level changes reconstructed in El Tobar agree with the quantitative signal of rainfall in Lake La Cruz (see Fig. 5), showing both a trend from drier towards more humid conditions over this period. Lower lake levels detected in El Tobar ca. AD 1715 and AD 1772 agreed well with a relatively dry period recorded in La Cruz ca. (1720-1780 AD) (Fig.4). High stand lake conditions recorded in El Tobar during the late LIA- Dalton Minimum ca. AD 1772-1850 are coincident with the two wettest periods in La Cruz (ca. AD 1770-1800 and 1815-1830). Finally, the 20th century appeared as a relatively wet period compared with the hydroclimatic conditions in the previous centuries.

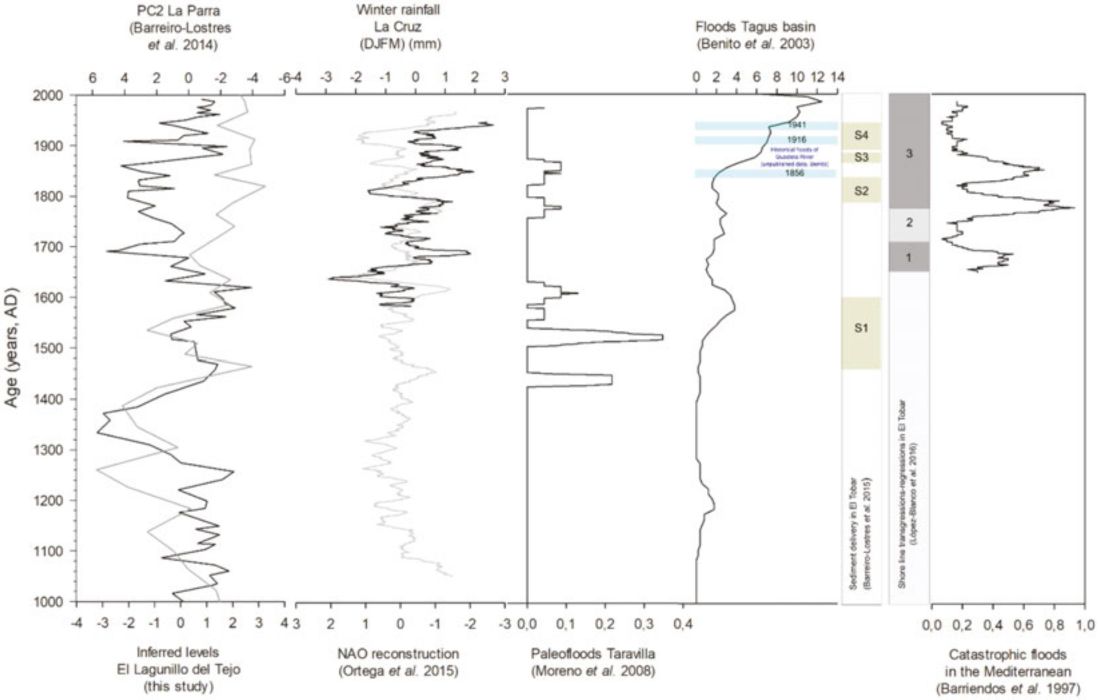

Figure 6. Compilation of inferences in La Cruz, El Lagunillo del Tejo and El Tobar with other paleoclimatic reconstructions at regional scale. From left to right: inferred levels in El Lagunillo (this study) and La Parra (Barreiro-Lostres *et al.*, 2014); the NAO (Ortega *et al.*, 2015) and Winter Rainfall reconstruction in La Cruz; paleofloods frequency in Taravilla Lake (Moreno *et al.*, 2008); floods in the Tagus basin (Benito *et al.*, 2003) and historical floods of Guadiela River (unpublished data, Benito); events of sediment delivery (Barreiro-Lostres *et al.*, 2015) and shore-line transgressions and regressions in El Tobar (López-Blanco *et al.*, 2016); catastrophic floods in the Mediterranean (Barriendos *et al.*, 1997). *Recopilación de las inferencias en La Cruz, El Lagunillo del Tejo and El Tobar con otras reconstrucciones paleoclimaticas a escala regional. De izquierda a derecha: niveles inferidos en el El Lagunillo (este estudio) y La Parra (Barreiro-Lostres* et al.*, 2014); reconstrucción de la NAO (Ortega* et al.*, 2015) y la precipitación de invierno en La Cruz; frecuencia de paleoavenidas en Taravilla (Moreno* et al.*, 2008); avenidas en la cuenca del Tajo (Benito* et al.*, 2003) y avenidas históricas en el río Guadiela (datos no publicados, Benito); eventos de aporte sedimentario (Barreiro-Lostres* et al.*, 2015) y trasgresiones y regresiones de la línea litoral de El Tobar (López-Blanco* et al.*, 2016); avenidas catastróficas en el Mediterráneo (Barriendos* et al.*, 1997).*

The climate signal recorded through calcite annual lamination in Lake La Cruz was analysed in detail in Romero-Viana *et al.* (2011). The presence of 0.12 and 0.25/year periodicities in this regional rainfall reconstruction, which are similar to those observed for NAO index series (Hurrell, 1995), confirmed a strong correlation with this large-scale pattern. The current comparison between calcite varves signal and the NAO index reconstruction from Ortega *et al.* (2015) showed higher correlations over the whole millennium, and a decoupling signal during shorter time periods (Fig. 4), than previous comparisons with three other NAO index reconstructions (Romero-Viana *et al.*, 2009). Ortega *et al.* (2015) have performed a yearly NAO reconstruction for the past millennium, based on an initial selection of 48 annually resolved proxy records distributed around the Atlantic Ocean, and built it through an ensemble of multivariate regressions. Unlike a previous NAO reconstruction by Trouet *et al.* (2009), Ortega *et al.* (2015) showed no persistent positive NAO during the MCA but more frequently positive values during AD 1270-1320. Our regional reconstruction from central-eastern Spain does not support the positive values of the NAO index during the MCA either, and showed a highly qualitative agreement with Ortega *et al.* (2015). Although the chronological model from El Lagunillo in the bottom of the core CN-4 is well constrained by four consecutive AMS ^{14}C dates, this chronology is far from showing annual resolution, and it is possible that the low lake levels recorded in El Lagunillo from ca. AD 1280-1400 could correspond to the NAO signal between ca. AD 1270-1320, where more positive values were recorded (Ortega *et al.*, 2015) (Fig. 4).

Another proxy that supports the existence of specific drier periods that favoured the occurrence of natural fires in the last millennium is the macrocharcoal content. In Lagunillo del Tejo, there was a continuous occurrence throughout the sedimentary sequence (Fig. 4). However, two main peaks were observed in ca. AD 1200-1250 and between AD 1800-1900, and two minor peaks were registered between ca. AD 1390 and AD 1525 (Fig. 4). The first two peaks of charcoal were most probably related to wars between Christians and Muslims, and also to the upsurge in sheep raising ("Mesta") based on transhumance (Lozano-Sahuquillo, 2002). The other two minor peaks were synchronous with dry periods in this reconstruction but also agree well with other local activities that could have generated human-made fires (López-Blanco *et al.*, 2012a). In El Tobar, the charcoal concentration was higher than in El Lagunillo. However, they were concentrated in a specific period of time ca. AD 1675 until AD 1820. This period agrees well with lower lake levels, but also with abundant historical information that relates fires to the collapse of the transhumance in the northern part of la Serrania de Cuenca (Bacaicoa *et al.*, 1993). As López-Blanco *et al.* (2012a) have argued previously, there were obvious socio-economical causes behind the fire history in this highly anthropogenic landscape, however, fires were probably favoured by drier conditions, showing the interplay between climate and human activity in the area.

When comparing our hydroclimate regional signal with other Iberian reconstructions, we found that the MCA is one of the most controversial periods. As mentioned before, arid conditions are inferred during this period in northeastern and southern IP, (e.g. Basa de la Mora, Estanya, Arreo and Zoñar) (see review of Moreno *et al.*, 2012) but sedimentary sequences situated in the western and central Iberia like this study indicated relatively wetter conditions for this key period. Small discrepancies between these inferences may be partially due to the influence of different climatic circulation patterns over a territory as complex as the IP. Recent studies suggest that other modes of variability than the NAO, such as the Eastern Atlantic or the Scandinavian (SCAND) patterns, significantly affect climate in the IP (Jerez & Trigo, 2013). Sánchez-López *et al.* (2016) published a review where they discussed the role of the NAO on humidity patterns in Iberian precipitation, and found homogeneous spatial climate conditions dominating the MCA and LIA in the IP. They discovered N-S and E-W humidity gradients acting during the Roman Period or the Early Middle Ages, however, as a result of the predominant coincidence of the NAO and East Atlantic index (EA) in opposite phases. On the other hand, Hernandez *et al.* (2015) demonstrated that the NAO is responsible for winter precipitation while the EA governs winter and summer temperatures, which agree very well with the hydroclimatic reconstruction in La Cruz and NAO relationship. It is therefore possible that the inclusion of more well-dated and multiproxy sequences in future spatial analysis in the IP will reveal new gradients or patterns in the hydroclimatic variability and its relationship with different modes of variability.

CONCLUSIONS

This study shows that the three karstic lakes described above preserved a valuable hydroclimatic signal in their sediments. The fact that this climatic signal was inferred not only by a single proxy, but by a combination of geochemical and biological indicators in different lacustrine systems, enhances the reliability of the reconstruction, shows the complexity of paleolimnological interpretations and highlights the importance of neolimnological knowledge in understanding the sedimentary inputs. Our results offer interesting and new information about the hydroclimatic conditions during the MCA in central eastern Spain. We have inferred relatively humid and highly variable conditions from ca AD 1000-1300, and this is strongly supported by new findings on NAO variability during the last millennium. Located in a key region under the influence of the NAO, the climatic reconstruction of these small sensitive systems could provide valuable information about this atmospheric circulation mode. Longer and higher resolution sediments in these and other permanent sinkholes in La Serranía de Cuenca could serve to quantitatively calibrate the different proxies, extending and improving this reconstruction back in time and thus, placing current tendencies into a longer-term context.

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Mid-Holocene and historical palaeoecology of the Albufera de València coastal lagoon

Marco-Barba, J.[1], Burjachs, F.[2,3,4], Reed, J.M.[5], Santisteban, C.[6], Usera, J.M.[6], Alberola, C.[6], Expósito, I.[3,4], Guillem, J.[6], Patchett, F.[5], Vicente, E.[1], Mesquita-Joanes, F.[1,*] and Miracle, M.R.[†]

[1] Cavanilles Institute of Biodiversity and Evolutionary Biology, University of Valencia. Catedrático José Beltrán Martínez, 2. 46980 Paterna, Spain.
[2] ICREA, Pg. Lluís Companys 23, 08010 Barcelona, Catalonia, Spain.
[3] IPHES, Institute of Human Palaeoecology and Social Evolution. Campus Sescelades URV, edifici W3. 43007 Tarragona, Catalonia, Spain.
[4] URV, University Rovira i Virgili, Àrea de Prehistòria. Av. de Catalunya 35. 43002 Tarragona, Catalonia, Spain.
[5] Dept. of Geography and Geology, School of Environmental Sciences, University of Hull. HU6 7RX Kingston-upon-Hull, UK.
[6] Dep. of Botany and Geology, University of Valencia. Av. Dr. Moliner, 50. 46100 Burjassot, Spain.
[†] University of Valencia (deceased).

[*] Corresponding author: mezquita@uv.es

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ABSTRACT

Mid-Holocene and historical palaeoecology of the Albufera de València coastal lagoon

The Albufera de València coastal lagoon is one of the largest oligohaline lagoons in the Iberian Peninsula. Highly polluted and threatened by plans for urban development, it has been protected as a Natural Park since 1986 to preserve its environment and surroundings, mostly consisting of ricefields and a forested coastal sand bar. Restoration plans focus on recovering the water quality and submerged macrophyte cover that occupied most of the lagoon in the 1950's. Until recent studies, little was known about the wetland's palaeoenvironmental history. To improve this knowledge, we analysed the Holocene evolution of the lagoon based on sedimentology, geochemistry and microfossils (foraminifera, diatoms, ostracods and pollen remains) from four cores. Two were collected in the sand bar, and two from the central lagoon. In combination with previous work, our new data show that the lagoon remained brackish for most of its history since 8700 cal BP, with the frequent presence of accompanying freshwater taxa from 7000 to 3400 cal BP. Notwithstanding chronological uncertainties, some episodes of decline in the abundance of microfossils seem to match aridity events Bond 5 (8.2 ky BP) and Minorca 7 (7.5-7.2 ky BP), the latter marking the switch from a dominance of arboreal vegetation to grasses. The most important change in the water body consisted of a sharp change at the beginning of the 19th century from a brackish to an oligohaline lagoon, driven by anthropogenic hydrological control associated with the expansion of ricefields. Later on, by the 1960-1970's, the growing population impacts of agricultural, wastewater and industrial effluents launched a major eutrophication process that would eventually sharply reduce the benthic vegetation and invertebrate communities and promote the phytoplankton dominance of the ecosystem in a turbid state. Although our multiproxy study has increased understanding of the lagoon's history, somewhat supported by documentary evidence, further palaeoecological research in different parts of the wetland would help define the causes of heterogeneous timing of changes in this large, shallow, complex system. Notwithstanding the need for further research, there is a clear priority for managers and the society to work on restoration efforts to drive the Albufera wetland towards one of the previous, less impacted, states of this worn-out and neglected ecosystem.

Key words: Palaeolimnology, pollen analysis, NPP, diatoms, Ostracoda, Foraminifera, isotopes, XRF, climatic events, coastal lagoon evolution, Iberian Peninsula

RESUMEN

Paleoecología de la laguna costera de la Albufera de València durante el Holoceno medio y en época histórica

La laguna costera de la Albufera de València es uno de los mayores lagos oligohalinos de la Península Ibérica. Fuertemente contaminada y amenazada por planeamientos de desarrollo urbanístico, fue finalmente protegida como Parque Natural en 1986, con el fin de preservar la cubeta y sus ambientes aledaños, los cuales consisten fundamentalmente en arrozales y un bosque sobre la barra arenosa costera. Los planes de restauración se centran en recuperar la calidad del agua y la cobertura de macrófitos sumergidos que ocupaban la mayor parte de la laguna en los años 50. Hasta la publicación de algunos trabajos recientes, se conocía bien poco de la historia paleoambiental esta zona húmeda. Con el fin de mejorar esta falta de conocimiento, estudiamos la evolución de la laguna durante el Holoceno mediante análisis sedimentológicos, geoquímicos y de microfósiles (foraminíferos, diatomeas, ostrácodos y restos polínicos) de cuatro registros. Dos de ellos fueron obtenidos en la barra arenosa, y otros dos del centro del lago. En combinación con trabajos anteriores, nuestros nuevos resultados muestran que la laguna permaneció como un ambiente salobre durante la mayor parte de su historia desde 8700 cal BP, con presencia frecuente de taxones dulceacuícolas acompañantes entre los 7000 y 3400 cal BP. A pesar de la incerteza de la cronología, se observan algunos episodios de reducción de abundancia de microfósiles acuáticos que parecen corresponderse con los eventos de aridez Bond 5 (8.2 ky BP) y Menorca 7 (7.5-7.2 ky BP), este último coincidiendo con un cambio notable de dominancia de vegetación arbórea a herbácea. El cambio mayor en el sistema acuático consistió en una transformación drástica a principios del s. XIX de una laguna salobre a una oligohalina, producida por un control antropogénico de la hidrología, asociado a la expansión del cultivo del arroz. Más tarde, entre 1960 y 1970 aproximadamente, el incremento poblacional y los impactos asociados de la agricultura, las aguas residuales y los efluentes industriales provocaron un proceso extraordinario de eutrofización que conllevaría una fuerte reducción de la vegetación sumergida y las comunidades de invertebrados, promoviendo la dominancia del fitoplancton de un sistema que pasó a un estado de alta turbidez. Aunque nuestro estudio con múltiples indicadores haya aumentado nuestro conocimiento de la historia de la laguna, con la ayuda de evidencias documentales, un mayor esfuerzo de investigación paleoecológica en diferentes zonas del humedal ayudaría a definir las causas de una dinámica temporal heterogénea en este extenso y complejo humedal. A pesar de esta necesidad de nuevas investigaciones, actualmente existe una prioridad para los gestores y la sociedad, de trabajar en esfuerzos de restauración que lleven a la zona húmeda de la Albufera hacia uno de los estados previos menos impactados de este ecosistema ampliamente sobreexplotado y desatendido.

Palabras clave: *Paleolimnología, análisis polínico, palinomorfos no polínicos, diatomeas, Ostracoda, Foraminifera, isótopos, XRF, eventos climáticos, evolución de lagunas costeras, península ibérica*

INTRODUCTION

> *"València té els matins lluents,*
> *pel sol que juga amb el mar*
> *i corre per l'Albufera"*
> Al-Russafí de València (12th century)
> version by J. Piera (1983)

As poets have sung for centuries, the Albufera lagoon is recognized as a keystone symbol of landscape beauty in the area of València and, in modern parlance, is of very high value as an 'ecosystem service' in the semi-arid Mediterranean climate. As a scarce and valuable water resource, it has long been threatened by intensive agricultural, industrial and demographic development, however, which has ultimately had a severe impact on the aquatic ecosystem and its surrounding catchment. Its current status is one of a highly polluted and artificially-regulated water body, mostly used as a dam for ricefield irrigation and as a deposit for sewage outflow (Vicente & Miracle, 1992; Soria, 2006). An urban development plan designed in the 1960's for the transformation of a forested area on the sand bar between the lagoon and the sea caused widespread environmental protest and resulted in the designation of the wetland ecosystem as a Natural Park in 1986 (Fundació d'Amics del Parc Natural de L'Albufera, 2000). In spite of this, the sad story of the ongoing process of eutrophication and degradation of water quality, and recent attempts at restoration, are well documented (Soria, 2006, Miracle *et al.*, 2012). Knowledge of a lagoon's pre-impact state is essential for effective restoration, but almost no data are available either on limnology or landscape biodiversity prior to the first quarter of the 20th century, particularly for ages previous to the publication of the work by Cavanilles (1795).

What was the origin of the lagoon? What was the impact of the marine transgression following the Pleistocene-Holocene climatic transition? Was its (pre-) historical limnology similar to observations of the first half of the 20th century by Pardo (1942)? Archaeological and historical sources point to a very long history of human occupation around the Albufera wetland system, with extensive resource exploitation, providing evidence which suggests growing impacts since early prehistorical times up to drastic habitat modification and desiccation of large areas of the lake during the past few centuries (Mateu *et al.*, 1985; Rosselló i Verger, 1995; Sanchis Ibor, 2001).

The first palaeolimnological work in the lagoon was carried out by Margalef & Mir (1973), who found diatom-based evidence for a marked salinity decrease in the top 20 cm of two short cores. Similar differences between surface and deeper (up to 85 cm) sediment samples from the bottom of the lagoon were also found when analysing the malacological composition (Robles *et al.*, 1985), with a remarkable abundance of the lagoon cockle *Cerastoderma glaucum* (Bruguière, 1789) in the deeper zones of the three short cores extracted. These authors conclude that the lagoon was previously brackish, in contrast to the present-day freshwater (oligohaline) state. Considering the scarcity of palaeoecological studies since these works (but see recent research by Carmona *et al.*, 2016), and keen to understand the evolution of the wetland ecosystem, the senior author (MRM) of the present text launched a multi-proxy project in 2002. From this effort, papers have been published on the stratigraphy and evolution of the Albufera, mostly based on ostracod remains (Santisteban *et al.*, 2009; Marco-Barba *et al.*, 2012; 2013a; b), but much information remains unpublished. In this paper we strengthen previous inferences by incorporating additional proxy data for the history of the lagoon and its catchment, including pollen, foraminifera and diatoms. These new proxy data support and build up on the palaeohydrological interpretations previously provided only by lithology and Ostracoda remains from two littoral cores and, most importantly, also add new landscape palaeoenvironmental reconstructions based on pollen analysis, since the end of the early Holocene. A new core from the centre of the lake is thoroughly analysed using high resolution XRF analysis and ostracod community and geochemical data to compare it with previous records from other areas of the lake and therefore contribute to unveil the spatial coordinated or heterogeneous response of the lake to human impacts and environmental changes. Furthermore, the new core expands the time period previously analysed for a shorter core (which encompassed from the 18th to 20th century), providing a wider time window down to earlier Middle Ages.

Previously, the origin of the lagoon was thought to date to the maximum marine Holocene transgression at c. 6000 y BP (Mateu *et al.*, 1985; Ruíz & Carmona, 2005), but our results to date (Santisteban *et al.*, 2009; Marco-Barba *et al.*, 2013a), and those of recent publications (Carmona *et al..*, 2016), suggest that a brackish wetland was already present by 8700 cal. BP, whose enclosing sandbar was probably located to the east of the present-day coastline. This earlier origin is somehow in agreement with the ontogeny of other active coastal lagoons since c. 8000 BP in Southern France (Mateu *et al.*, 1985). Other coastal lagoons appeared since at least 7400 BP in the south of the Iberian Peninsula (Almeria; Goy *et al.*, 2003), or since 7500-7600 cal. BP in the nearby sites of Elx (Blázquez & Usera, 2010) and Almenara, with a previous brackish marsh in Almenara during the earlier drier period (Blázquez *et al.*, 2017). Furthermore, the Mesolithic remains of *Cerastoderma glaucum* (not dated but considered older than 7000 BP) found in the adjacent hills to the west of the present Albufera de València (Mateu *et al.*, 1985) also support such early origin. It is difficult to say to what extent prehistoric societies might have modified the landscape, other than hunting and gathering for food. Palaeopalynological records from other Mediterranean coastal areas of the Iberian Peninsula suggest evidence for anthropic effects as early as the Boreal period (8000-9000 y BP), and presence of *Olea* and *Vitis*, added to inland deforestation during the following millennium (López-Sáez & López-García, 1999). Later, at c. 7500 cal. BP, the archaeological remains in the region indicate the beginning of the Neolithic agricultural and pastoral practices (Bernabeu *et* *al.*, 2014, Bernabeu-Aubán *et al.*, 2016). Recent, historical human impacts on the landscape and the limnology of the lagoon have been severe, but it might be possible that they started long ago. Here we try to unveil part of the long history of the lagoon and infer climatic and human effects on its long-term dynamics.

METHODS

Study area

The Albufera de València (Fig. 1) is one of the largest oligohaline lagoons of the Iberian Peninsula. It has an area of 2320 Ha with a mean depth

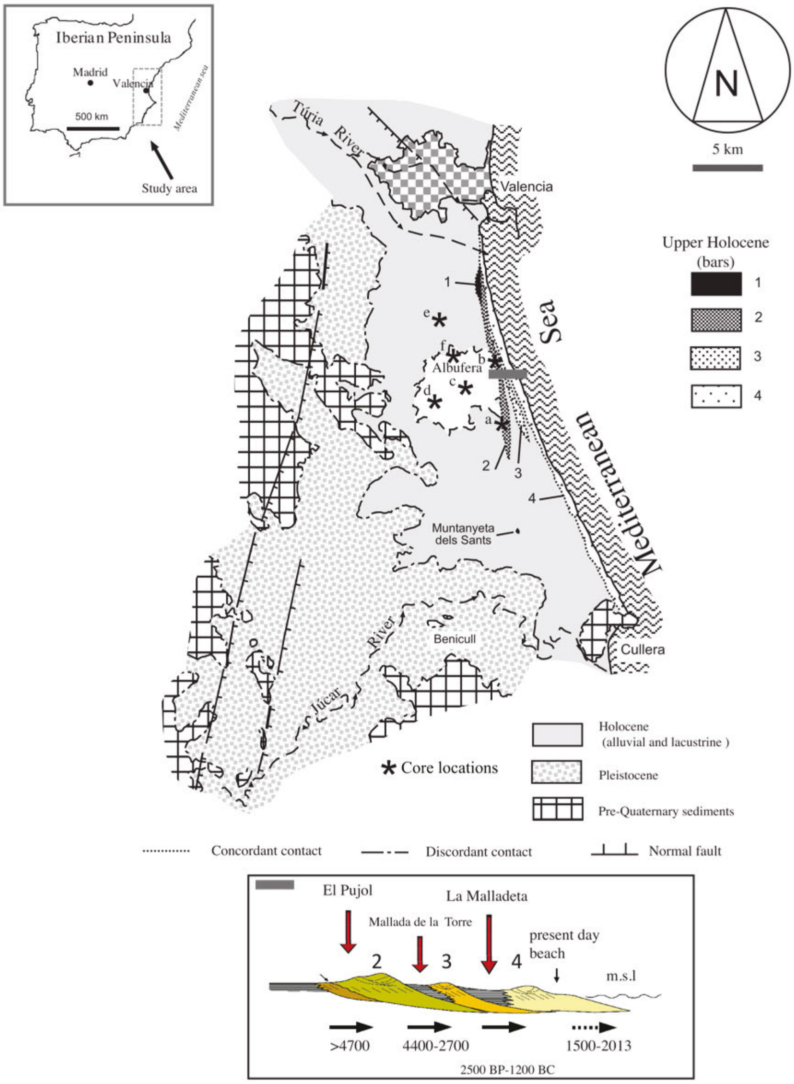


Figure 1. Geological and lithological composition of the studied area in Albufera de València, showing the location of the cores analysed or discussed in the text. A lithological transversal cut of the sand bar is also shown (after Santisteban *et al*, 2009). Core locations: a, Core Palmar; b, Core Pujol; c, Core Centre; d, Core Antina; e, core in Carmona *et al.* (2016); f, core in Rodrigo *et al* (2010). *Composición geológica y litológica del área de estudio en la Albufera de València, así como localización de los registros estudiados o discutidos en el texto. Se muestra también un corte transversal de la barra arenosa (a partir de Santisteban* et al.*, 2009). Localizaciones de los registros: a, Core Palmar; b, Core Pujol; c, Core Centre; d, Core Antina; e, registro en Carmona* et al. *(2016); f, registro en Rodrigo* et al. *(2010).*

of 1 m, separated from the sea by a sand bar (≈1 km wide). It is enclosed on the Quaternary floodplain lying between the alluvial fans of Túria (North) and Xúquer/Júcar (South) rivers. These rivers drain the southeastern sector of the Iberian Mountain System (mostly composed of Cretaceous and Jurassic limestones); the precipitation regime is characterized by a marked seasonal pattern, such that peak flash floods may reach 3000 m^3/s. Although the fluvial hydrology is modified by various dams, the rivers retain the capacity to transport gravels, silt and clay sediment to the lower reaches. The Túria River sediment load contributed to the formation during the Holocene of the sand bar that protects the Albufera from the sea (Carmona & Ruiz, 1999). The sediments are transported by coastal drift currents towards the south and deposited by wave action during seasonal eastern storms. According to Santisteban *et al.* (2009), the sand bar is composed of four prograding units (Fig. 1). The oldest one (pre-4700 y BP) is moderately eroded. The second and the third have the configuration of spit beaches developed at the southernmost part of the Holocene Túria delta, and the last unit was formed in historical times.

Climatic and climate-driven events such as sea-level change, storm events and river floods have affected the Albufera's hydrology, ecology, water chemistry composition and landscape throughout its history. With accelerated impacts likely to have been initiated by the development of irrigated rice paddies and commercial and population expansion during the Industrial Revolution (towards the mid 19th century), the Albufera de València is currently a hypereutrophic and highly polluted lagoon (Vicente & Miracle, 1992; Marco-Barba *et al.*, 2013a,b).

Core material and dating

Four sediment cores were extracted from the Albufera lagoon (Fig. 1). Two long sequences were collected in the sand bar: El Palmar, 18.5 m long, and Pujol, 16 m long. The extraction methods, lithology and ostracod palaeoecology and chronology for the El Palmar core are described by Santisteban *et al.* (2009) and Marco-Barba *et al.* (2013a), encompassing a period from 8700 to 3400 cal y BP. The same methods of extraction and ostracod analysis apply to core Pujol, and these and its lithological description, in relation to core El Palmar are also included in Santisteban *et al.* (2009). Unfortunately, no radiocarbon dates are available for core Pujol, but Santisteban *et al.* (2009) provide a stratigraphical correlation with core El Palmar. The remaining two, shorter, cores were extracted from the inundated central part of

Table 1. Radiocarbon dates obtained for Core Centre, and material and laboratory details. The last two columns indicate the estimated (est.) calendar years from sediment accumulation rates and other proxies (historic data, magnetic susceptibility and XRF analysis), and the corresponding reservoir effect (R(t)) calculated from the estimated dates. See text for further explanations. *Dataciones de radiocarbono obtenidas del core Centre, y detalles del material y del laboratorio de análisis. Las últimas dos columnas indican la edad en años calendario estimada (est.) a partir de las tasas de acumulación del sedimento y otros indicadores (datos históricos, susceptibilidad magnética y análisis XRF), así como el efecto reservorio (R(t)) correspondiente, calculado a partir de las fechas estimadas. Véase el texto para mayor explicación.*

Depth (cm)	^{14}C date (uncal. yr BP)	^{14}C date (cal. yr BP)	Material	Laboratory	Lab. Code	Est. date (cal. yr AD)	R(t) (yr)
89	1460 ± 30	995 ± 65	shell	Beta Analytic	313444	1772	817
94	1340 ± 30	875 ± 55	shell	Beta Analytic	313445	1744	669
135	2655 ± 30	2794 ± 55	pollen	Poznań Radioc. Lab.	Poz-19788	1450	2294
140	2400 ± 30	2030 ± 80	shell	Beta Analytic	313446	1400	1480
225	3090 ± 30	2850 ± 80	shell	Beta Analytic	313447	550	1450
229	3520 ± 35	3789 ± 100	pollen	Poznań Radioc. Lab.	Poz-18865	510	2349
233	22260 ± 140	22265 ± 285	pollen	Poznań Radioc. Lab.	Poz-18288	470	n.d.

the lagoon. The lithology, chronology, ostracod palaeoassemblages and geochemistry of the shortest (63 cm long encompassing the early 18th to the end of the 20th century) core Antina were presented by Marco-Barba *et al.* (2013b). Finally, a 240 cm core (Core Centre) was taken in July 2006 from a point in the middle of Albufera de València (UTM: 30S728065/4357504; Fig. 1). It was extracted using a Livingstone corer with 8 cm diameter inner liner and kept in a polyethylene tube. The core was analysed at 0.5 cm resolution for magnetic susceptibility (MS) with a GEOTEK core logger at the Limnological Research Center (LRC) of the University of Minnesota (USA), and was then subdivided in two halves. One half was used for imaging (DMT core scanner) colour, grain-size descriptions and ITRAX XRF core scanning; the other half was sampled for fossil content and geochemical analysis.

For Core Centre, seven radiocarbon dates (Table 1) were obtained from mollusc shells (*Cerastoderma glaucum*) and pollen concentrates selected from subsamples with high organic matter content. The radiocarbon measurement (´BP´) was calibrated to calendar years (´cal.BP´) using OxCal v3.10 software, with the Intcal04 curve for pollen and the Marine04 curve for shells (Hughen *et al.*, 2004; Reimer *et al.*, 2004; Bronk Ramsey, 2005) and later corrected for the reservoir effect in aquatic environments (see details in the results section). To determine age and sediment accumulation rates for the past 200 years a twin short (1 m) gravity core was extracted in the same location for ^{210}Pb-^{137}Cs dating. ^{210}Pb was measured at 4-6 cm depth intervals by means of its granddaughter product ^{210}Po (Eakins & Morrison, 1976). Activity was measured for 1-6 x 10^5 s with ion-implanted surface barrier detectors and an Ortec alpha spectroscopy system. The ^{137}Cs values were confirmed by gamma spectrometry on an EG&G Nuclear ultra-low background well detector. All dating analyses were performed at Minnesota’s St. Croix Watershed Research Station. Unsupported ^{210}Pb was calculated by subtracting supported activity from the total activity measured at each level; supported ^{210}Pb was estimated from the asymptotic activity at depth (the mean of the lowermost samples in the core). Dates and sedimentation rates were determined according to the cf:cs. (constant flux: constant sedimentation model, Appleby and Oldfield, 1978) with confidence intervals calculated by first-order error analysis of counting uncertainty (Binford, 1990).

XRF and geochemical analysis

The main geochemical elements Al, Si, P, S, Cl, K, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Rb, Sr, I, Zr, Ba and Pb were analysed every 5 mm from the bottom to 66 cm depth and every 2 mm from 66 cm to the top of Core Centre on the ITRAX XRF core scanner at the Large Lakes Observatory of the University of Minnesota (Duluth, USA) using 30 sec count time, 30 kV X-ray voltage and an X-ray current of 20 mA. The obtained values are given as counts per second, although high correlations have been found between ICP-OES and XRF-scanner data for some elements in different studies (Boyle, 2000; Moreno *et al.*, 2010). The XRF results for each element were transformed to arcsine of the square root of proportions; the data matrix was analysed by means of PCA (Principal Components Analysis).

The subdivision of Core Centre was carried out at approximately 2 cm intervals for the bottom metre and every 1 cm (high resolution) in the upper metre. In total, 72 samples were obtained for different analysis (geochemistry, mineralogy and micropalaeontological studies). Aliquot subsamples (between 0.3-0.7 g) were weighed, dried at 105 ºC during 2 h and weighed again to determine water content and dry weight per gram of fresh sample. The remaining sediment was heated at 460 ºC for 7.5 h to determine organic matter content (loss on ignition; LOI) and subsequently at 950 ºC for 8 h to obtain the concentration of carbonates per sample, calculated from CO_2 loss (Heiri *et al.*, 2001). Subsamples were also taken to measure total nitrogen (TN) and phosphorus (TP) content. Samples were digested according to Valderrama (1981) modified for sediments. All samples were also submitted to a conventional digestion procedure with sulphuric acid and potassium persulphate for phosphorus analysis (APHA, 1992; Ferree & Shannon, 2001).

Diatom analysis

To assess limnological variation in the littoral and profundal zone, and for comparison with vegetation history, the El Palmar (long edge core) and lagoon centre (Antina) sequences were selected for analysis. Diatoms were prepared using standard techniques (Battarbee *et al.*, 2001), mounting slides with Naphrax®. Where diatoms were preserved, El Palmar samples were counted initially at ca. 10-12 cm resolution and at higher resolution (min. 3 cm) in zones of good preservation, comprising 65 samples. A full count (> 500 valves) was possible in 12 subsamples and diatom species presence was noted for a further 8 samples in which diatoms were rare. A full count was possible in 13 samples prepared at approximately 4 cm resolution (range 2 – 8 cm) in the upper 53 cm of the Antina sequence, with rare preservation below. Diatoms were identified using Krammer & Lange-Bertalot (1986, 1988, 1991a and 1991b), adopting updated diatom nomenclature. Counts were converted to percentage data and displayed using Tilia (Grimm, 1991-2015). Zone boundaries were defined using constrained incremental sum of squares analysis (CONISS; Grimm, 1987). Diatom-conductivity reconstruction was carried out based on the European Diatom Database Combined Salinity training set (Battarbee *et al.*, 2000), using weighted averaging with inverse deshrinking in the programme C2 (Juggins, 2007), and data from this and the Spanish diatom-conductivity training set (Reed, 1998) were used to aid interpretation. The abundance of fragilarioid taxa (facultative planktonic group), which have very broad ecological tolerance ranges, is problematic for quantitative reconstruction, so these taxa were omitted in application of the transfer function to El Palmar.

Foraminifera analysis

Foraminifera remains were analysed from a total of 62 samples from El Palmar core, 64 samples from Pujol, 12 samples from the Antina sequence and 49 samples from Core Centre. Sediment samples were air dried and then submersed in warm water to separate the organic matter from foraminifera. No other chemical products were used, to avoid damaging agglutinated foraminiferal shells, which are common in lagoonal environments. Samples were then sieved under a smooth water flow using a 62 μm mesh. The remains were dried prior to analysis under the stereomicroscope. A minimum number of 300 individuals per sample were picked for species determination and counting. The data were analysed for diversity changes over time using the effective number of species approach (Jost, 2006), by means of diversity of order 0 (S = species richness) and order 1 (e^H = exponential of Shannon diversity using natural logarithms).

Ostracod analysis

Subsamples from core Centre were processed for ostracod analysis using a modified version of Griffiths & Holmes (2000), and explained in detail in Marco-Barba *et al.* (2013a,b) for cores El Palmar and Antina. Similarly, for Core Centre, between 10 and 15 g of wet sediment were weighed, air dried and then wet-sieved for 30 minutes through 400 and 250 μm pore size meshes. Species were identified following mostly Athersuch *et al.* (1989), Meisch (2000) and Poquet *et al.* (2008). We noted the states of preservation (SP) of shells as described in Marco-Barba *et al.* (2013a). Only valves belonging to good preservation states (A and B) were included in multivariate analysis (see below).

Between 1 and 137 *Cyprideis torosa* adults per sample (adult females and males) were measured for carapace length, as this is known to potentially vary with salinity (Mezquita *et al.*, 2000). The percentage of noded animals per sample (van Harten, 1975; Marco-Barba, 2013a) was also calculated to assess salinity variation (Vesper, 1972a,b; van Harten, 1975; Marco, 2010; Frenzel *et al.*, 2012; Marco-Barba *et al.*, 2013a).

Between four and 20 *C. torosa* valves were analysed per sample of Core Centre for shell oxygen and carbon isotopic variation. Analyses were performed on single adult valves by preference; in the absence of these, juveniles of late growth instars (A-1 and A-2) were pooled in groups of 4 or 5 valves. Adults and juveniles, and males and females, were analysed separately.

Between two and 10 analyses were carried out per sample to determine the valve calcite isotopic ratios $^{18}O/^{16}O$ and $^{13}C/^{12}C$. Ostracod valves were cleaned for geochemical analysis using the method described in Ito (2001). Cleaned valves were analysed mostly using a Kiel-II online carbonate preparation device coupled to a Finnigan MAT 252 stable isotope ratio mass spectrometer (University of Minnesota) and a few samples (60 samples≈159 valves) using a MAT 251 (Michigan) mass spectrometer, both connected to a Kiel carbonate preparation device. Analytical precision was typically < 0.1 ‰ for both $\delta^{13}C$ and $\delta^{18}O$. Samples were run from the same levels in the two different labs to ensure that the different analytical protocols did not affect the results.

The number of valves per 10 g of dry sample (N = 70 samples) was log transformed ($\log_{10}$ (number of valves+1)) for ostracod stratigraphical analysis. A constrained cluster analysis was performed applying the CONISS method and the Edwards & Cavalli-Sforza distance (Grimm, 1987). The whole transformed data matrix was ordered with PCA.

Correlations between *C. torosa* shell lengths and isotope ratios ($\delta^{18}O_{vpdb}$ and $\delta^{13}C_{vpdb}$) were performed using either Pearson (when data was normally distributed) or Spearman (for not normally distributed data) correlation techniques. Palaeosalinity reconstruction of lagoon Albufera from Core Centre was carried out using ostracod-based weighed averaging transfer functions based on the species' optima and tolerances as detailed in Marco-Barba *et al.* (2013b).

Palynological analysis

To provide a long record of vegetation history, with high resolution analysis of recent change, pollen analysis was carried out in two selected sequences – the long core, El Palmar, and the short central core, Antina. Subsamples were selected to span the range of lithological zones in El Palmar (62 samples) and at 1 cm resolution (53 samples) in Antina. The preparation of pollen, non-pollen palynomorphs (NPPs) and palynological microcharcoal samples followed standard methods outlined in Goeury & Beaulieu (1979) but adapted by Burjachs *et al.* (2003). The sediments were reduced by HCl, humic acids washed in NaOH, floated in dense Thoulet liquor, and filtered with a glass fibre filter which was subsequently destroyed with 70 % HF. All steps were followed by washing and centrifuging with distilled water. The final residue of each sample was mounted in preparation for biological microscopy diluted with glycerin. *Circa* 150-500 pollen grains of terrestrial taxa were counted using an Olympus CX41 microscope fitted with x15 oculars and x40/60 objectives. For El Palmar, results are presented for 33 samples in which a representative count was possible; pollen preservation was poor in the remaining 29 samples.

Cyperaceae, *Typha/Sparganium*, *Polygonum* t. *persicaria*, *Ruppia*, *Potamogeton*, *Myriophyllum* and *Lemna*-type have been excluded from the pollen sum to avoid overrepresentation by local littoral taxa. All pollen types are defined according to Moore *et al.* (1991), Reille (1992, 1995, 1998), Beug (2015) and Cerealia-type was defined according to the morphometric criteria of Andersen (1978), Faegri & Iversen (1989) and Tweddle *et al.* (2005). Non-pollen palynomorph (NPP) identification followed van Geel (1978, 2001), van Hoeve & Hendrikse (1988) and Revelles *et al.* (2016). To calculate the percentages of NPPs an independent basic sum of the pollens has been made. Pollen concentration (PC) is expressed in pollen grains/g of dry sediment following the volumetric method (Loublier, 1978). Pollen and NPPs were compiled and plotted using Tilia (Grimm, 1991–2015). Pollen and NPPs zones were established using the stratigraphically constrained square root transformation (CONISS) (Grimm, 1987).

RESULTS

Cores El Palmar and Pujol

The long, littoral sedimentary sequences of Palmar and Pujol were described by Santisteban *et al.* (2009). Further information on the chronology, ostracod palaeoecology, geochemistry and palaeoenvironmental interpretation of El Palmar was provided by Marco-Barba *et al.* (2013a). Usera *et al.* (1990; 1995; 2006; 2007a,b) and Usera & Alberola (2011) also provide brief notes

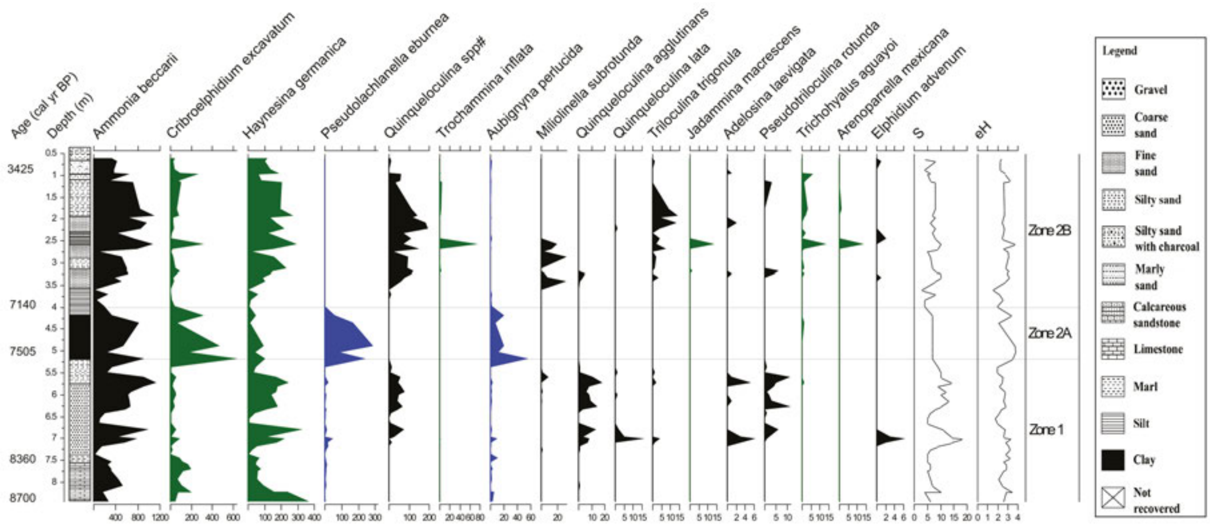


Figure 2. Summary stratigraphic diagram of foraminifera abundances in Lake Albufera core Palmar showing taxa present at minimum > 5 individuals. Green: species tolerant of relatively low salinity in brackish waters (other than *Ammonia* spp.); blue: those considered less tolerant to low salinity. The diversity of order 0 (S = species richness) and order 1 (e^H, where H = Shannon diversity), and zone boundaries defined by constrained cluster analysis are shown on the right. *Gráfico estratigráfico resumen de los foraminíferos del core Palmar de la laguna de la Albufera, mostrando las especies con un mínimo de individuos > 5. Verde: especies tolerantes a salinidades relativamente bajas en aguas salobres (aparte de* Ammonia *spp.); azul: aquellas poco tolerantes a bajas salinidades. A la derecha se muestran la diversidad de orden 0 (S = riqueza de especies) y orden 1 (e^H, donde H = diversidad de Shannon), así como las zonas definidas mediante análisis constreñido de clasificación.*

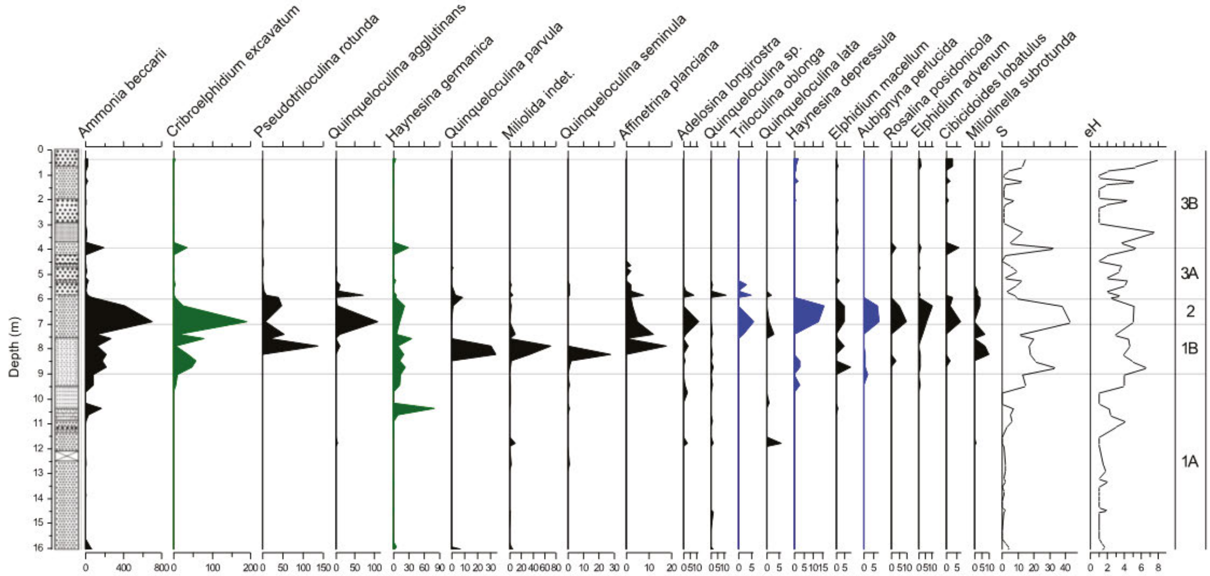


Figure 3. Summary stratigraphic diagram of foraminifera abundances in Lake Albufera core Pujol showing taxa present at minimum > 5 individuals. Green: species tolerant of relatively low salinity in brackish waters (other than *Ammonia* spp.); blue: those considered less tolerant to low salinity. The diversity of order 0 (S = species richness) and order 1 (e^H, where H=Shannon diversity), and zone boundaries defined by constrained cluster analysis are shown on the right. Sediment legend as in figure 2. *Gráfico estratigráfico resumen de los foraminíferos del core Pujol de la laguna de la Albufera, mostrando las especies con un mínimo de individuos > 5. Verde: especies tolerantes a salinidades relativamente bajas en aguas salobres (aparte de* Ammonia *spp.); azul: aquellas poco tolerantes a bajas salinidades. A la derecha se muestran la diversidad de orden 0 (S = riqueza de especies) y orden 1 (e^H, donde H = diversidad de Shannon), así como las zonas definidas mediante análisis constreñido de clasificación. Leyenda de la columna sedimentaria como en la figura 2.*

on the foraminifera of these cores and other parts of the lagoon. Here we incorporate new and extended palaeoecological data from foraminifera, diatom and pollen analysis.

Foraminifera

A total of 46 927 foraminifera were identified from core El Palmar, belonging to 47 species. The most abundant species through the sequence were *Ammonia beccarii* (Linné, 1758), *Cribroelphidium excavatum* (Terquem, 1875) and *Haynesina germanica* (Ehrenberg, 1840) (Fig. 2). Three (sub-)zones could be distinguished based on constrained cluster analysis. Zone 1 (850-520 cm) was rich in species but dominated by the three aforementioned taxa, resulting in relatively low e^H diversity values. The base of zone 2 (subzone 2A, 520 - 400 cm) shows a marked change in species dominance, with reduced species richness (S) but important peaks of *C. excavatum*, *Pseudolachlanella eburnea* (d'Orbigny, 1839) and *Aubignyna perlucida* (Heron-Allen & Earland, 1913), promoting increased evenness and consequently high ecological diversity of order one (e^H). The upper subzone (subzone 2B, 400-50 cm) shows again relatively high and constant S and e^H values.

In Pujol, the identification of 5744 foraminifera individuals yielded a higher number of species, with a total of 93 different taxa. Again, *A. beccarii* was the dominant species, together with *C. excavatum*, *H. germanica* and *Pseudotriloculina rotunda* (d'Orbigny in Schlumberger, 1893) (Fig. 3). Furthermore, *Quinqueloculina agglutinans* (d'Orbigny, 1839) is also occasionally abundant. The basal 7 m (subzone 1A, 1600-900 cm) of the sequence show the lowest diversity, with an increase to higher values in the next subzone (1B; 900 – 700 cm depth), corresponding to mud and clay lacustrine deposits during a regressive period (Santisteban *et al.*, 2009). Foraminiferal diversity reaches maximum peaks in the following Zone 2 (700 – 600 cm depth), during a sandy beach phase in a transgressive period (Santisteban *et al.*, 2009). The upper zone (Zone 3; 600 – 50 cm) is variable in species composition, but shows a return to lower diversity and number of individuals collected, similar to subzone 1A.

Diatoms

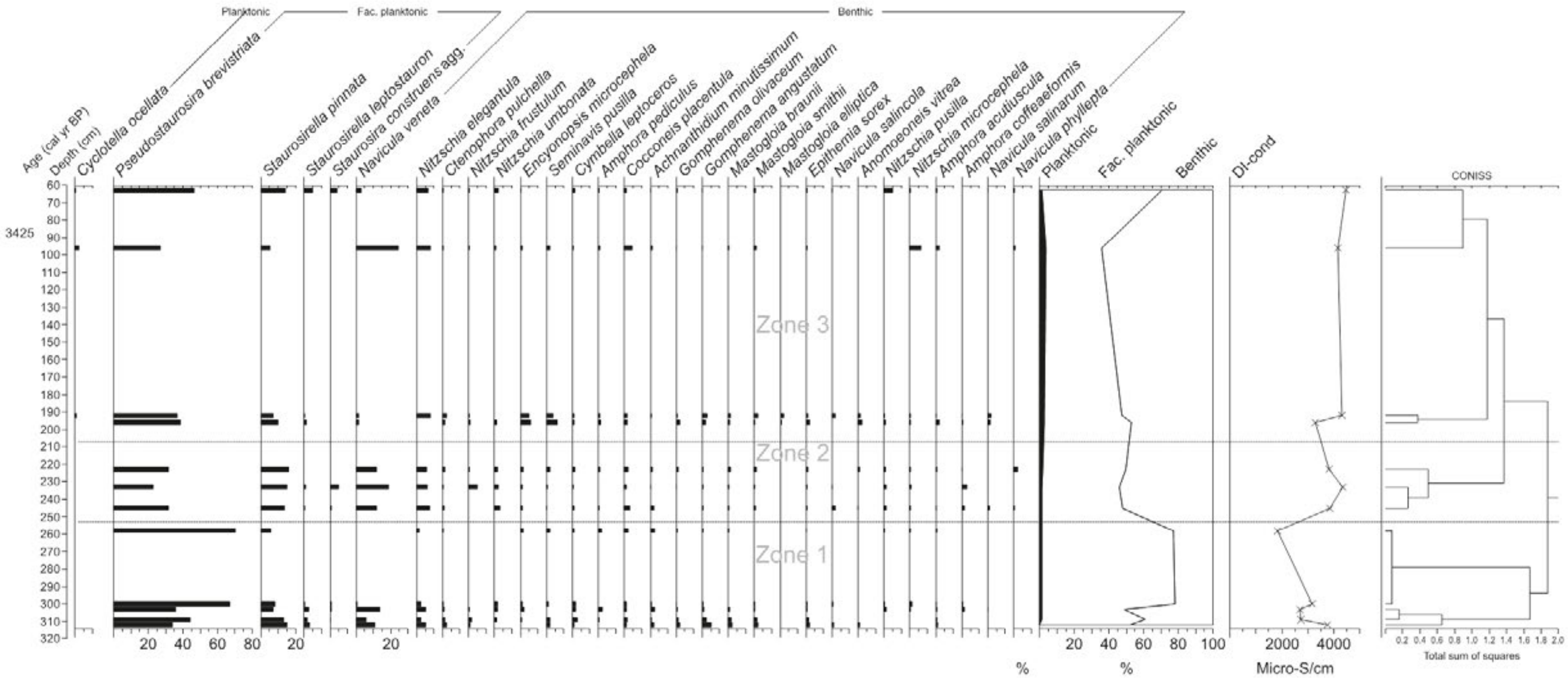
Diatoms from El Palmar (Fig. 4) are only preserved from 312 cm to 63 cm depth, from the mid Holocene onwards. Samples where diatoms were present but too sparse for a full count (99, 112, 178, 209, 269, 274, 288, 319, 326 and 341 cm depth) all contain robust taxa which are resistant to dissolution, but of a similar character to zones of better preservation. Three diatom zones can be recognised, but the sequence essentially shows little evidence for major limnological change. The sequence is dominated by benthic and facultative planktonic (FP) taxa throughout. Planktonic taxa are rare. The taxa are all common in the modern environment of Spanish saline lakes (Reed *et al.*, 2001) and are mainly those which tolerate slightly brackish (oligosaline) to mesosaline waters. This is reflected in the complacency of the diatom-inferred conductivity (DI-Cond) curve, which fluctuates around the oligo-mesosaline boundary of 3000 μS/cm.

Zone 1 (341.5 -251.5 cm) is dominated by FP taxa, and *Pseudostaurosira brevistriata* in particular. A range of benthic taxa, including obligate saline species such as *Mastogloia braunii* and *Amphora coffeaeformis*, are present at up to 50 % abundance. Planktonic taxa are present at < 2 %. The presence at low abundance of *Achnanthidium minutissimum*, a freshwater species common in streams, may indicate the influence of freshwater inflow to the lagoon. The uppermost sample exhibits minimum DI-Cond, a sample which is dominated to 80 % by FP taxa.

Zone 2 (251.5 – 209.5 cm) is marked by a reduction in the relative abundance of *P. brevistriata*, accompanied by slight increases in benthic taxa including *Navicula veneta*. Some obligate saline taxa in the Mastogloia genus decrease in abundance but the continued occurrence of others (*A. coffeaeformis*, *Nitzschia elegantula*, etc.) suggests no marked shift in limnology.

Zone 3 (209.5 – 63 cm) is a zone of particularly poor diatom preservation. The zone boundary is again marked by subtle shifts in diatom species assemblage composition, such as a slight increase in obligate, saline *Seminavis pusilla*. The first presence of plankton at > 2 % abundance at 96 cm depth is that of a *Cyclotella ocel-*

Figure 4. Summary stratigraphic diagram of diatom abundances in Lake Albufera core Palmar. The conductivity reconstruction (DI-cond) and dendrogram derived from constrained cluster analysis are shown on the right. *Diagrama estratigráfico de abundancias de diatomeas del core Palmar de la laguna de la Albufera. A la derecha se muestra la reconstrucción de la conductividad (DI-cond) y el dendrograma resultante de un análisis de clasificación.*



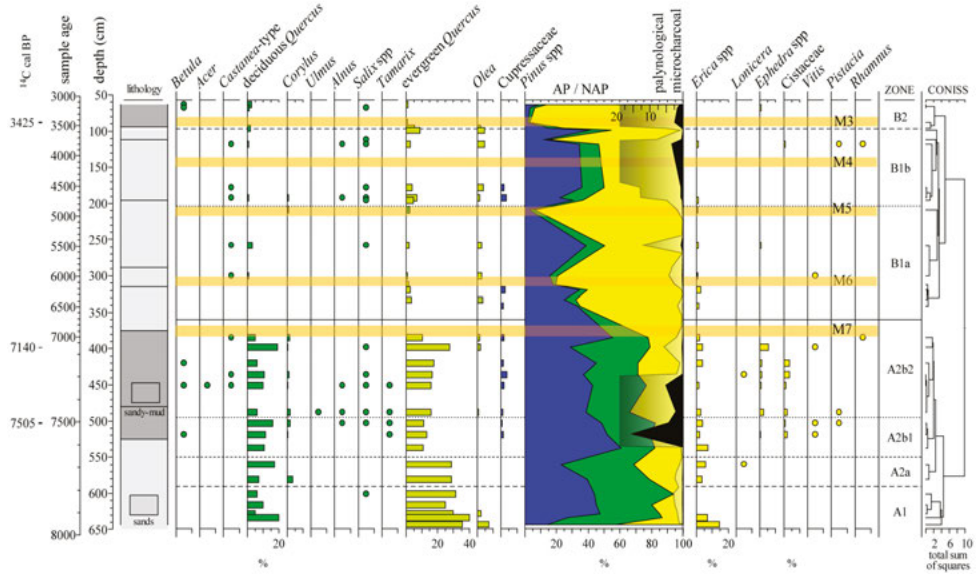


Figure 5. Stratigraphic diagram of pollen percentages (trees and shrubs) and palynological microcharcoal in Lake Albufera core Palmar. The black microcharcoal palynological curve corresponds to the real values (x10000), while its degraded plot corresponds to an exaggeration x20. The zones defined by constrained cluster analysis are shown on the right. The dots mean less than 1 %. The orange bands mark the Minorca events (Frigola *et al.*, 2007). *Diagrama estratigráfico de porcentajes polínicos (árboles y arbustos) y microcarbones palinológicos del core Palmar del lago de la Albufera. La curva negra de los microcarbones palinológicos corresponde a los valores reales (x10000), mientras que el gráfico sombreado corresponde a una exageración x20. A la derecha se muestran las zonas establecidas por el análisis de clasificación constreñido. Los puntos indican valores < 1 %. Las bandas anaranjadas corresponden a los eventos de Menorca (Frigola* et al.*, 2007).*

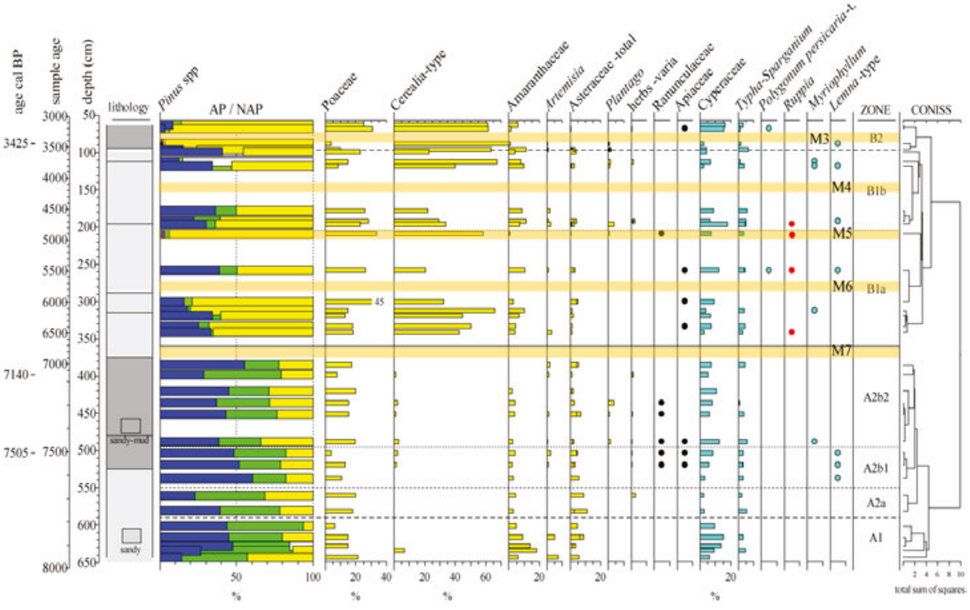


Figure 6. Stratigraphic diagram of pollen percentages of herbs in Lake Albufera core Palmar. The zones defined by constrained cluster analysis are shown on the right. The dots mean less than 1 %. The orange bands mark the Minorca events (Frigola *et al.*, 2007). *Diagrama estratigráfico de los porcentajes de polen de hierbas en el core Palmar de la laguna de la Albufera. A la derecha se muestran las zonas definidas por un análisis de clasificación constreñido. Los puntos indican valores < 1 %. Las bandas anaranjadas corresponden a los eventos de Menorca (Frigola* et al.*, 2007).*

lata, a freshwater taxon of broad tolerances which can withstand low salinity.

Pollen and NPP

In general, the lower half of the sequence is richer in species and total pollen abundance, allowing greater resolution, whereas the top 350 cm show lower abundance and even barren samples (Figs. 5-8).

The vegetation of Zone A (643-380 cm, Fig. 5) is dominated by *Pinus* spp. and *Quercus* spp., representing an open forest with scrubs, which include mainly *Erica* spp., Cistaceae, *Pistacia* sp., with some presence of *Ephedra* sp. suggesting sand dune habitats (Fig. 5). There is a smooth declining trend of the arboreal pollen towards the top of the sequence, but arboreal pollen abundance is almost always above 70-80 %. Riparian vegetation is scarcely represented, with presence of *Corylus* sp., *Alnus* sp., *Ulmus* sp., *Salix* spp. and *Tamarix* spp. Probably originating from the surrounding mountain areas, some pollen from *Betula* sp., *Acer* sp., *Castanea*-type and deciduous *Quercus* sp. is observed also in this zone. At the bottom of the zone (643-600 cm, zone A1), the high relative abundance of Amaranthaceae (Fig. 6) indicates saline influence in the wetland area; Cyperaceae also occurs, with an approximately negative covariation with Amaranthaceae, suggesting a heterogeneous and variable wetland with variation in soil salinity. From this period on, the Amaranthaceae decline, but are present throughout this zone, while Cyperaceae remains more stable.

Unlike pollen remains, non-pollen palynomorphs (NPPs) are present from the bottom of the sequence (c. 8600 cal. BP at 850 cm) (Figs. 7, 8). The base of the sequence (850-760 cm, subzone A1a) is poor in NPPs, but at the top of this subzone (A1b) the percentages of megaspores (Fig. 7) are remarkably high. The rest of zone A (700-350 cm) is more diverse (Figs. 7, 8), including mainly algae (*Gloeotrichia*), fungal (hyphae) and undetermined microzoological remains (type 52).

The upper Zone B (340-63 cm) shows a large shift of vegetation towards a major reduction in arboreal cover, to less than 50 %. Wild grasses (Poaceae) increase up to c. 40 %, and Cerealia-type show an exceptional expansion to above 50 %. We think that this pollen comes from dune grasses, since we know that some of these species have a size of grain as large as the cultivated cereals (e.g. Joly *et al.*, 2007; Farooq *et al.*, 2015). Besides the major change at c. 340 cm, other notable events of a sharp reduction in arboreal pollen occur at 310, 210 and 90 cm depth.

Regarding NPPs, Zone B exhibits higher diversity and abundance of fungi and algal remains than Zone A, with a clear increase of *Botryococcus* spp. (Fig. 7). Polyporisporites also increase in abundance (Fig. 8) but megaspores disappear from this zone. The presence of eggs of parasitic worms (Nematoda) is notable throughout this zone. Charcoal peaks tend to correspond to maximum values of AP, when there is more forest mass available to burn (Burjachs & Expósito, 2015).

Core Antina

Marco-Barba *et al.* (2013b) previously described the chronology, sedimentology and ostracod palaeoassemblages and geochemistry of this sequence, generating the first palaeoenvironmental interpretation of the sequence. Here we add new data on pollen, foraminifera and diatom palaeoassemblages to improve the reconstruction of the recent history of the lake and its surrounding landscape.

Foraminifera

Only ten species of foraminifera (Fig. 9) were found in this short core (8373 individuals counted), and they were present only at the bottom part (70-50 cm). The species *Ammonia tepida* (Cushman, 1926), *C. excavatum* and *H. germanica* dominated, and were the only species present at the top samples (Zone 2) that contained foraminifera. There is a remarkable peak in abundance of *Trichohyalus aguayoi* (Bermúdez, 1935) at 55 cm, just when most species have disappeared or show low mean abundances.

Diatoms

Diatoms (Fig. 10) are well preserved throughout, apart from at the start of the sequence, below 60 cm. The sample at 60 cm only contained

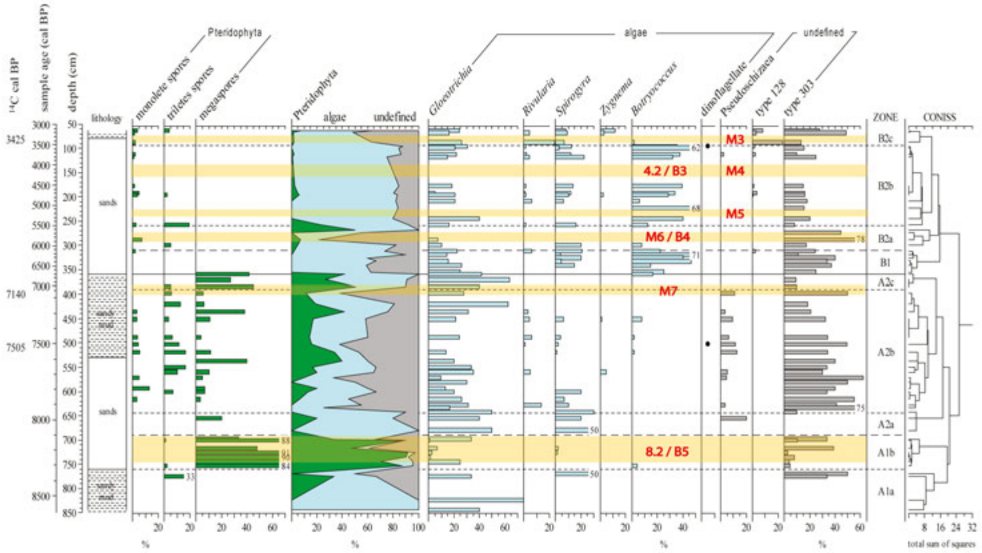


Figure 7. Stratigraphic diagram of Pteridophyta spores, algae and undefined NPPs percentages in Lake Albufera core Palmar. The zones defined by constrained cluster analysis are shown on the right. The dots mean less than 1 %. The orange bands mark the Minorca (Frigola *et al.*, 2007) and Bond events (Bond *et al.*, 1997). *Diagrama estratigráfico de los porcentajes de esporas de Pteridophyta, de algas y de NPPs no definidos en el core Palmar de la laguna de la Albufera. A la derecha se muestran las zonas definidas por un análisis de clasificación constreñido. Los puntos indican valores < 1 %. Las bandas anaranjadas corresponden a los eventos de Menorca (Frigola* et al.*, 2007) y eventos Bond (Bond* et al.*, 1997).*

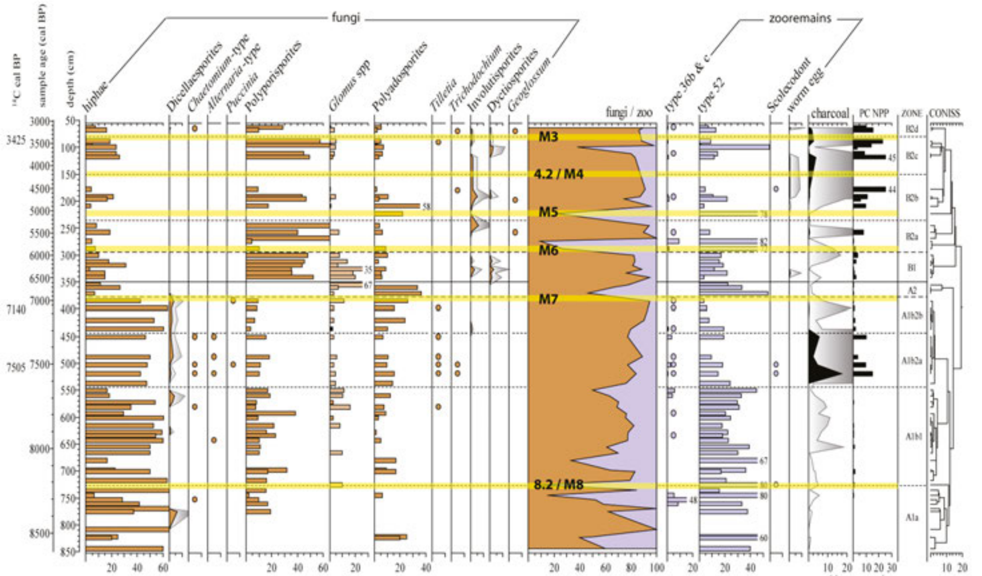


Figure 8. Stratigraphic diagram of fungi, zooremains NPPs percentages, absolute frequencies of palynological charcoal and total concentration of NPPs in Lake Albufera core Palmar. The zones defined by constrained cluster analysis are shown on the right. The dots mean less than 1 %. The yellow bands mark the Minorca (Frigola *et al.*, 2007) and Bond events (Bond *et al.*, 1997). The curves exaggeration factor is variable, in order to better visualize its evolution. *Diagrama estratigráfico de los porcentajes de hongos, restos de NPPs zoológicos, frecuencias absolutas de carbones palinológicos y concentración total de NPPs en el core Palmar de la laguna de la Albufera. A la derecha se muestran las zonas definidas por un análisis de clasificación constreñido. Los puntos indican valores < 1 %. Las bandas amarillas corresponden a los eventos de Menorca (Frigola* et al.*, 2007) y eventos Bond (Bond* et al.*, 1997). El factor de exageración de las curvas es variable, para poder visualizar mejor su evolución.*

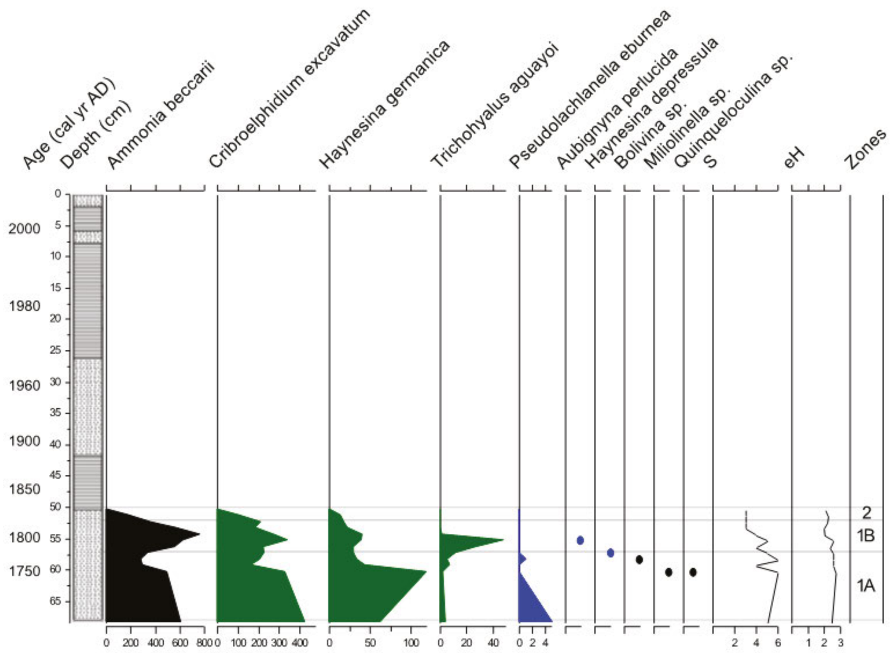


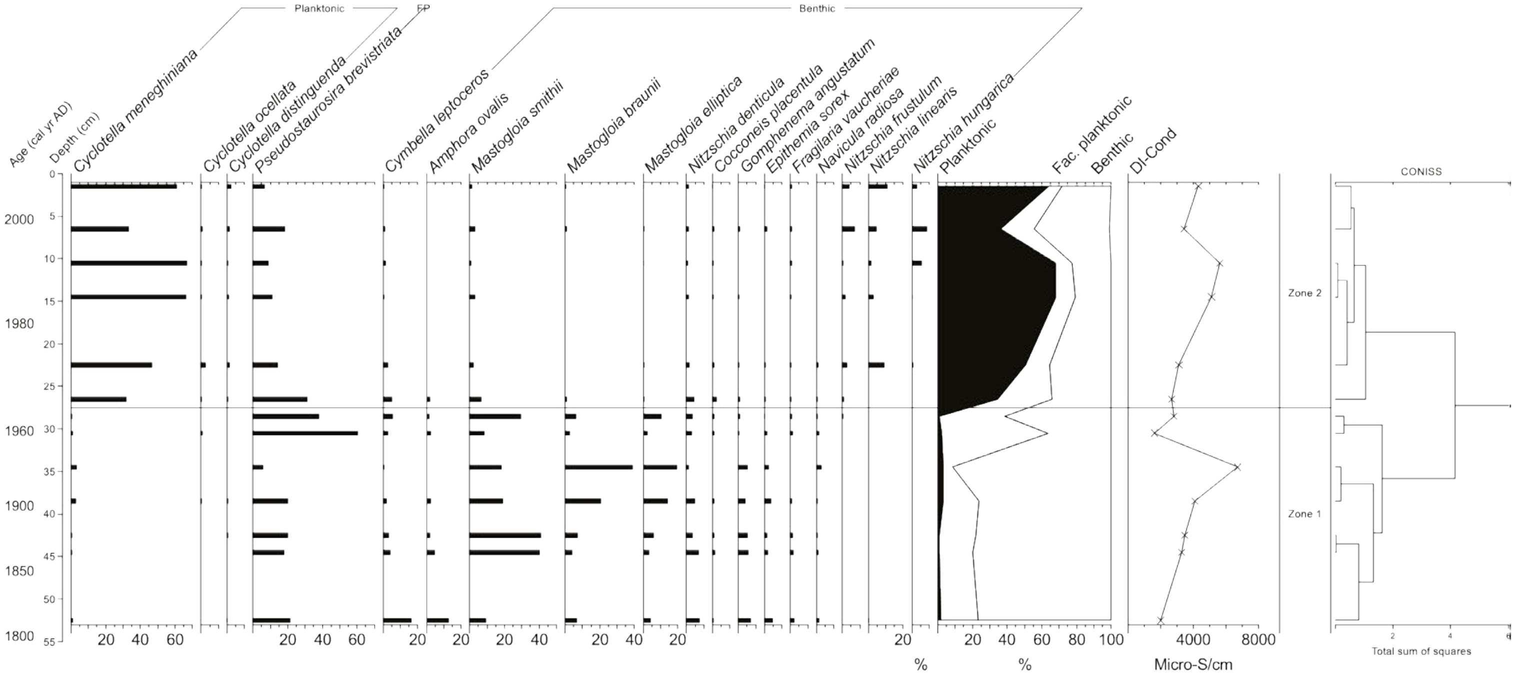
Figure 9. Summary stratigraphic diagram of foraminifera abundances in Lake Albufera core Antina showing taxa present at minimum > 5 individuals. Green: species tolerant of relatively low salinity in brackish waters (other than *Ammonia* spp.); blue: those considered less tolerant to low salinity. The diversity of order 0 (S = species richness) and order 1 (e^H, where H = Shannon diversity), and zone boundaries defined by constrained cluster analysis are shown on the right. Sediment legend as in figure 2. *Gráfico estratigráfico resumen de los foraminíferos del core Antina de la laguna de la Albufera, mostrando las especies con un mínimo de individuos > 5. Verde: especies tolerantes a salinidades relativamente bajas en aguas salobres (aparte de* Ammonia *spp.); azul: aquellas poco tolerantes a bajas salinidades. A la derecha se muestran la diversidad de orden 0 (S = riqueza de especies) y orden 1 (e^H, donde H = diversidad de Shannon), así como las zonas definidas mediante análisis constreñido de clasificación. Leyenda de la columna sedimentaria como en la figura 2.*

fragments of two species, *Campylodiscus clypeus* and *Diploneis bombus*, both of which indicate high conductivity. Above, the sample at 56 cm is more diverse, preserving some species not present in other dissolved samples: *Epithemia argus* and *Amphora libyca* (in addition to *D. bombus* and *M. braunii*), but again indicates high salinity (Ben Khelifa, 1989; Reed, 1998).

Zone 1 (52.5 – 27.5 cm) is dominated by *P. brevistriata* and a similar range of brackish taxa were present at lower abundance in the lake edge sequence. The dominant benthic taxa, *Mastogloia smithii* (fresh-brackish) *M. braunii* (obligate saline) and *M. elliptica* (obligate saline) are typical both of Spanish salt lakes and chloride-dominated estuarine/lagoonal waters. Plankton are represented at < 5 % abundance by *Cyclotella meneghiniana*, a species common in shallow waters (1-2m depth) of Spanish salt lakes (Reed, 1998). DI-cond tends to fall in the mesosaline range of > 3000 µS/cm. A trend towards increased relative abundance of *M. braunii* and *M. elliptica* at the expense of the less salt-tolerant *M. smithii* is matched by peak DI-Cond of c. 7000 µS/cm at 34.5 cm depth; FP taxa increase towards the upper zone boundary, with an associated reduction in DI-Cond to its minimum for the sequence, in the oligosaline range.

The transition to Zone 2 (27.5 – 0.5 cm) is marked by a sharp increase and subsequent dominance of planktonic *C. meneghiniana* at the expense of FP and the range of benthics present in Zone 1. A range of *Nitzschia* taxa (*N. frustulum*, *N. linearis* and *N. hungarica* appear for the first time. *C. meneghiniana* is again a taxon of very broad tolerance. It is a common diatom of saline waters, but also of hypereutrophic or generally polluted fresh water (Mills & Ryves, 2012). Its high conductivity optimum drives the mainte-

Figure 10. Summary stratigraphic diagram of diatom percentages in Lake Albufera core Antina. The conductivity reconstruction (DI-cond) and dendrogram derived from constrained cluster analysis are shown on the right. *Diagrama estratigráfico de porcentajes de diatomeas del core Antina de la laguna de la Albufera. A la derecha se muestra la reconstrucción de la conductividad (DI-cond) y el dendrograma resultante de un análisis de clasificación.*



nance of DI-Cond at c. 4000 µS/cm, but this may be overestimated. The increase in *Nitzschia* taxa at the expense of *Mastogloia* points to a marked reduction in salinity.

Pollen

Subzone A1a: 61-55 cm

The percentage of arboreal pollen (AP) is relatively high (c. 40-80 %) at the base of the sequence, dominated by *Pinus* (pines), *Quercus* evergreen and *Q.* deciduous (Holm oak, oaks), *Olea-Phillyrea* (olive tree, wild olive, green olive tree) and Cupressaceae (Fig. 11). The shrub component is most represented by heathers (*Erica*). The relatively high abundance of Amaranthaceae and Artemisia, the scarcity of freshwater algae and low values of hygro-hydrophytes (Cyperaceae, *Lemna*-type, *Typha-Sparganium*, *Myriophyllum*), all point to the influence of salinity (Fig. 12).

Subzone A1b: 55-46 cm

Here, there is a general reduction in AP despite increasing tree diversity. While the bushes remain stable, wild grasses (Poaceae) and Cerealia-type pollen (up to 5.5 %) increase. The highest abundances of Faboideae and Asteraceae are noted in this subzone and the aquatic plant *Myriophyllum* onset its presence, although at low abundance. A small peak in Pseudoschizaea abundance, which is considered an NPP associated to erosion (Pantaleón-Cano *et al.*, 1996), is also noticeable (Fig. 13). This may indicate enhanced sediment input, maybe favoured by forest fires, as also suggested by a peak in microcharcoal at that time (Fig. 13).

Subzone A2: 46-38 cm

Forest vegetation (AP) recovers up to levels similar to those of subzone A1 (43-60 %). Pollen salinity indicators (Amaranthaceae) decrease even more now, and pollen from riparian trees (*Corylus*, *Alnus*), which have appeared in the previous subzone, now increase, as well as the relative abundance of algal remains and the aquatic plant *Myriophyllum*, suggesting fresher conditions of the water body. A peak of *Juglans* (walnut tree) and the presence of *Morus* (used to feed silk moth caterpillars) indicate changes in land use with more importance of the trees cultivation.

Subzone B1: 38-30 cm

This subzone marks the onset of a new vegetation regime (Figs. 11 and 12). Initially, the climactic vegetation (oaks) retreats in favour of conifers (pines and Cupressaceae). By the end of this subzone, arboreal vegetation in general declines (particularly for *Olea* sp. and *Quercus* sp.), with a concomitant increase in grasses (Poaceae, up to 30 %, Fig. 12). The marked decrease in the aquatic plant *Myriophyllum*, and increase in fungi (type Polyadosporites, zone A2 in Fig. 13) suggest higher turbidity, possibly associated with eutrophication. This process may be also related to the rise in *Cosmarium* algal remains (zone A2 in Fig. 13). Most probably, the littoral areas of the lake suffered temporary variations in inundation through time, favouring the increase in Cyperaceae and grasses (Fig. 12).

Subzone B2a: 30-17 cm

Pine forests increase at this zone, although there is a reduction at the top of the subzone, related to fires, as indicated by microcharcoal increase (zone B1 in Fig. 13). Riparian areas seem now dominated mostly by bulrush (cf. *Typha*) rather than by common reed (cf. *Phragmites* included in Poaceae). The transition between the previous zone and this one is indicated by the input of detritic sediments (*Glomus* and, later, the type Pseudoschizaea), at the same time that there is an increase in microalgae *Spirogyra*, *Botryococcus* and *Pediastrum*, as a response to a relative reduction of *Cosmarium*. There is evidence throughout for cultivation (Cerealia), with the presence of mulberry trees (*Morus* sp.) At about 25 cm deep, the first presence of Myrtaceae occurs, probably indicating an increase in *Eucalyptus* plantations or extension of *Myrtus* (common myrtle).

Subzone B2b: 17-6 cm

This subzone is very similar to the previous one, but with evidence for a slight recovery of the

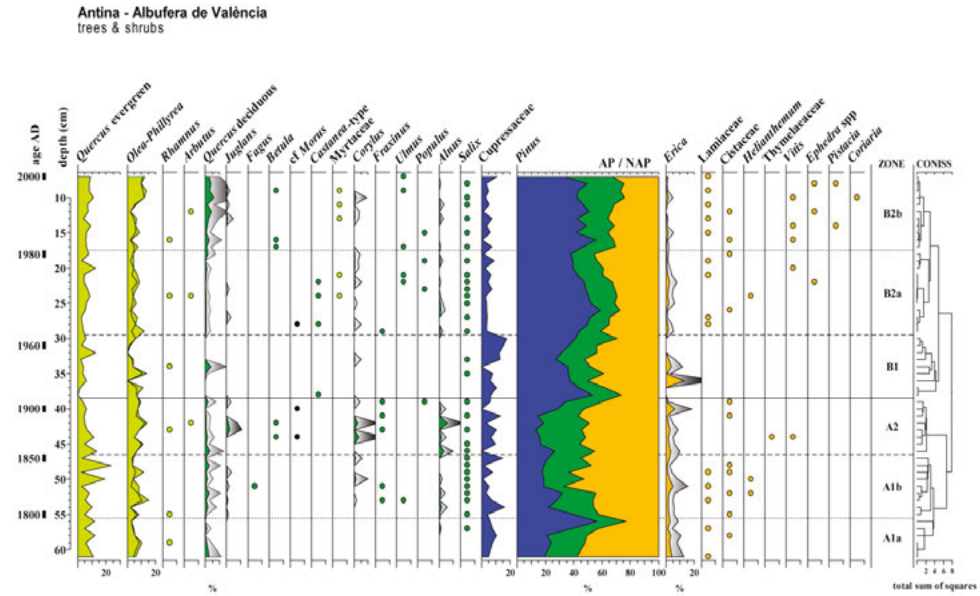


Figure 11. Stratigraphic diagram of pollen percentages in Lake Albufera core Antina. The zones defined by constrained cluster analysis are shown on the right. The dots mean less than 1 %. The curve exaggeration factors are variable, in order to better visualize their evolution. *Diagrama estratigráfico de los porcentajes de polen en el core Antina de la laguna de la Albufera. A la derecha se muestran las zonas definidas por un análisis de clasificación constreñido. Los puntos indican valores < 1 %. El factor de exageración de las curvas es variable, para poder visualizar mejor su evolución.*

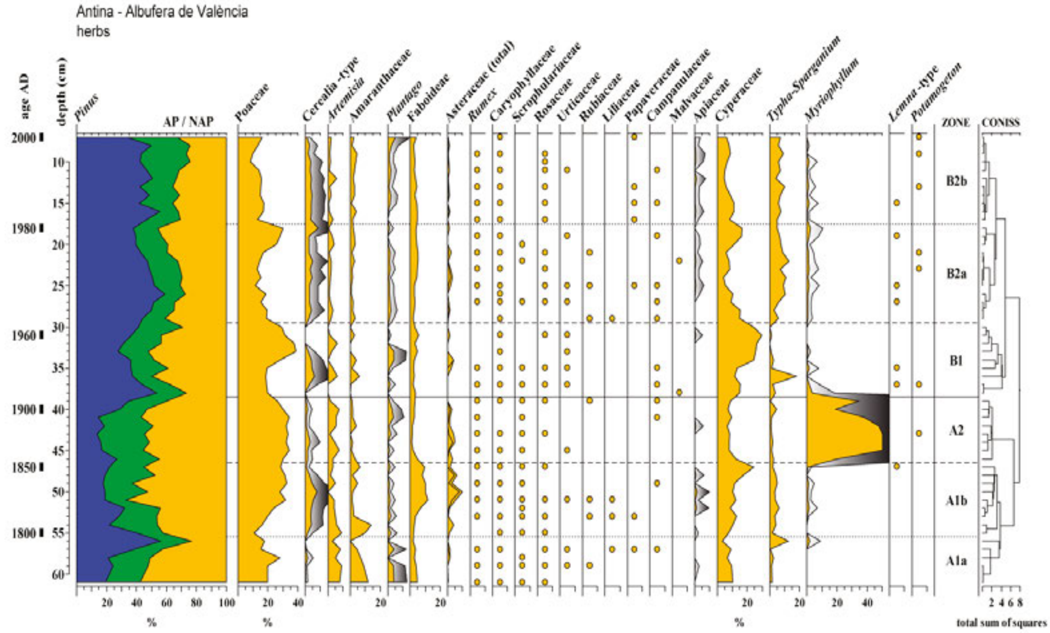


Figure 12. Stratigraphic diagram of pollen percentages of herbs in Lake Albufera core Antina. The zones defined by constrained cluster analysis are shown on the right. The dots mean less than 1 %. The curves exaggeration factor is variable, in order to better visualize their evolution. *Diagrama estratigráfico de los porcentajes de polen de hierbas en el core Antina de la laguna de la Albufera. A la derecha se muestran las zonas definidas por un análisis de clasificación constreñido. Los puntos indican valores < 1 %. El factor de exageración de las curvas es variable, para poder visualizar mejor su evolución.*

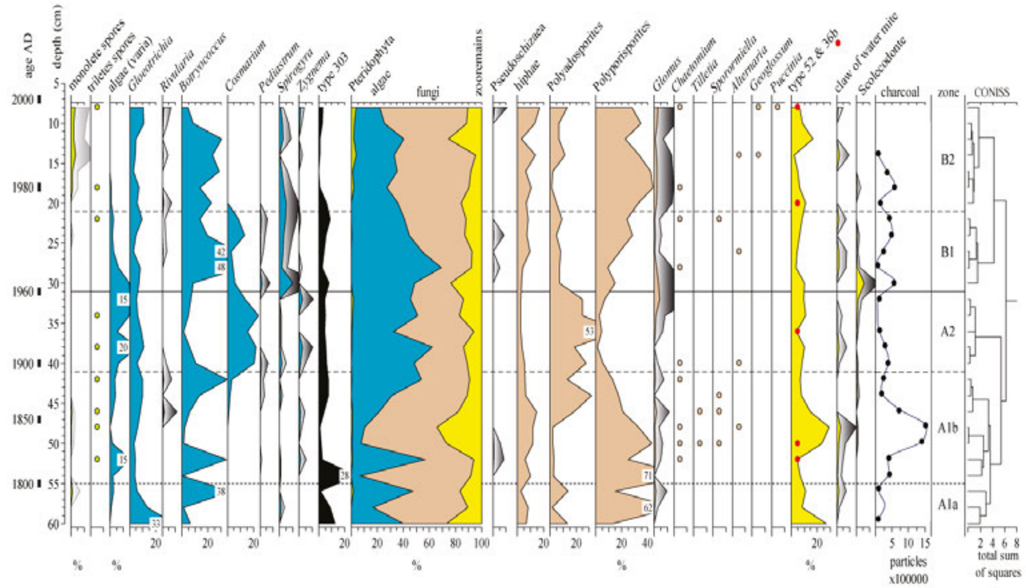


Figure 13. Stratigraphic diagram of Pteridophyta, algae, fungi, zooremain NPPs percentages and absolute frequencies of palynological charcoal in Lake Albufera core Antina. The zones defined by constrained cluster analysis are shown on the right. The dots mean less than 1 %. The curves exaggeration factor is variable, in order to better visualize their evolution. *Diagrama estratigráfico de los porcentajes de Pteridophyta, algas, restos NPPs zoológicos y frecuencias absolutas de carbones palinológicos en el core Antina de la laguna de la Albufera. A la derecha se muestran las zonas definidas por un análisis de clasificación constreñido. Los puntos indican valores < 1 %. El factor de exageración de las curvas es variable, para poder visualizar mejor su evolución.*

forest, along with the constant presence of Myrtaceae and cultivated cereals. No *Morus* remains are observed anymore. Freshwater taxa (Cyperaceae, *Typha/Sparganium*, cf. *Phragmites*, Pteridophyta monolete spores) continue to dominate over saline indicators (Amaranthaceae, *Artemisia*).

Core Centre

A brief summary of the top part of this core and its interpretation was previously given by Marco-Barba *et al.* (2012a). In this paper, we provide a detailed description of the stratigraphic sequence (Fig. S1, available at http://www.limnetica.net/en/limnetica), including the chronology (Fig. 14), information on chemical composition and a detailed XRF analysis of the sediments (Figs. 15, 16, S2 (available at http://www.limnetica.net/en/limnetica)), so as on foraminifera remains (Figs. S3 (available at http://www.limnetica.net/en/limnetica),17) and ostracod palaeoecology and geochemistry (Figs. S4 (available at http://www.limnetica.net/en/limnetica), 18, 19).

Chronological framework

For the top of the core a ^{210}Pb-based cf:cs chronology was constructed from the near-exponential section of the twin core (14-35 cm), which yielded dates that match the ^{137}Cs record fairly closely, giving a sedimentation rate of 6 mm/yr for the twin core Centre (Fig. 14), after lithological matching. This is supported by the very high magnetic susceptibility peak at the top of Core Centre (30 cm depth) that coincides with an important flood event in 1957, which markedly affected Albufera lake (Fig. S1; Carmona & Ruiz, 2000). Thus, the chronology presented here is arranged to fit the radiocaesium results, with the flood event as a tie marker. The year 1850 AD at the base of ^{210}Pb profile could be placed at 75 cm in core Centre, which fits with a change in the Sr/Ca content (Figs. 14,16) a shift of facies (top of *Cerastoderma* shells, Fig. S1) and coincides with a major expansion of the irrigation system at the beginning of the 19th century (Sanchis Ibor, 2001). A sedimentation rate of 4.2 mm/yr is

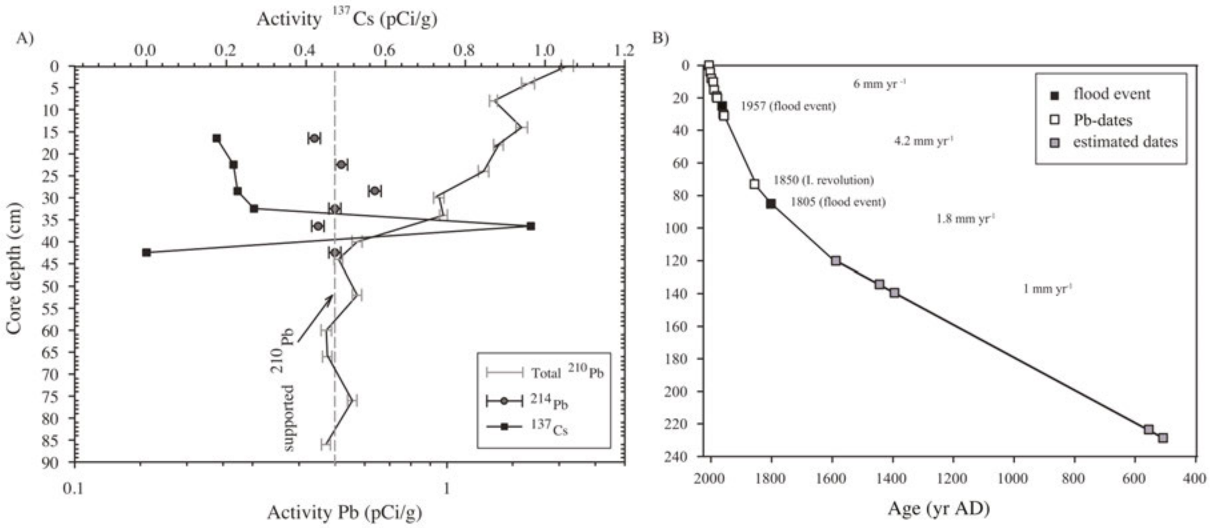


Figure 14. A) Radionuclide activities of ^{210}Pb (gray straight line), ^{214}Pb (gray circles) and ^{137}Cs (black squares) from Core Centre. Note that supported activity is also placed. B) Chronological model and sedimentation rates estimated from ^{14}C dates and historical data (see text). *A) Actividades (pCi/g) de los radioisótopos* ^{210}*Pb (línea gris),* ^{214}*Pb (puntos grises) y* ^{137}*Cs (cuadrados negros) del Core Centre. También se indica la actividad basal. B) Modelo cronológico y tasas de sedimentación estimadas a partir de dataciones* ^{14}*C y datos históricos (ver texto).*

estimated for the period 1850-1957 (Fig. 14). Just below that depth there are peaks in magnetic susceptibility and detrital elements (Figs. 15,16) that could be related to the very frequent acute storm episodes occurring in the wet years at the start of the XIX century (e.g. a very intense one in 1805; Carmona & Ruiz, 2000) involving again the rivers and water courses surrounding Albufera lake. So, we placed in our chronological model the year 1805 as corresponding to the magnetic susceptibility peak at 85 cm depth (Fig. 15) The peak could be additionally reinforced by the transportation of large volumes of sediment to transform the shallow peripheral parts of Albufera into rice paddies (increasing Ti/K) that took place mainly from the end of the 18th to the beginning of the 19th century. This chronology fits also with XRF results (Fig. 16); the Pb accumulation would increase after the start of the Industrial Revolution (starting between 1800-1850 AD in Europe and by the end of this period in Valencia, Renberg *et al.*, 2001; Bindler *et al.*, 2008), and Cu would increase with its use in the switch of rice cultivation from transplanting to direct seeding in the 1960's (Zaragoza Pérez, 1982).

The lower part of the chronological model (Fig. 14) is based additionally on radiocarbon dates (Table 1), which probably had a strong reservoir effect, but used to calculate sediment accumulation rate (SAR), plus historical data and XRF geochemistry. The reservoir effect can be very high and very variable (Sabatier *et al.*, 2010; 2012), especially when there is influence of freshwater rich in (bi-)carbonates, so that measured ^{14}C dates in such context can be up to > 2000 yr older than expected, and strongly depends on alkalinity (Coularis *et al.*, 2016). This effect can apply not only to shelled invertebrates, but also to aquatic plants with aerial parts (Philippsen, 2013; Philippsen & Heinemeier, 2013). Therefore, shell and pollen ^{14}C dates from lake Albufera sediments could be much older than the finally calibrated dates, taking into account the strong influence of freshwater input throughout its history (e.g. Marco-Barba *et al.*, 2013a,b), with variable and high alkalinity in recent times (Soria *et al.*, 1987). Anyway, the lower part of the core has a very stable Cl content, indicating insignificant anthropogenic changes in the hydrology of the lagoon, thus, a relative stability in the reservoir effect can be assumed, allowing average sediment accumulation rate (SAR) to be estimated from radiocarbon measurements of the same type of material at different depths. Average rates calculated independently from pollen and shell data for the lower part of the core showed similar values of around 1 mm/yr (SAR_{pollen}: 0.94-0.97 mm/yr and SAR_{shells}: 1.05-1.23 mm/yr). This was taken as the average SAR from the core base to 120 cm, after which a sharp Cl decrease occurs (Fig. 16). This shift in the Cl values is thought to be related to the construction of a gate in the outlet of the lagoon to the sea for fishing management in 1607, and consequently we assigned the date of 1600 yr AD to a core depth of 120 cm. This hydrological control, together with an increased drainage to this lagoon outlet from irrigation ditches, limited water interchange with the sea and reduced the salinity of the lagoon according to historical documents compiled by Sanchis Ibor (2001). Assuming that the depth of 120 cm corresponds to the first quarter of the 17th century, a SAR of 1.8 mm/yr could be assigned to the 75-120 cm interval (Fig. 14). These age estimates suggest that the results of shell analysis incorporate a reservoir effect (R(t)) much higher than the expected marine reservoir age in the Mediterranean Sea (about 400 years; Siani *et al.*, 2000), in accordance with observations in nearby coastal systems by Sabatier *et al.* (2010; 2012). This estimated effect was between 1450 and 2349 yr (Table 1) for our calibrated ^{14}C dates (excluding a much older outlier at 233 cm core depth), higher for pollen than for shell material. Due to the large uncertainty of the reservoir corrections and sedimentation of older materials, the radiocarbon results have therefore only been used as relative measurements, and those of the uppermost stratum (above 120 cm core depth) have been excluded, because deposition occurred in different and more variable hydrological conditions. Still, we estimated also a reservoir effect of 669-817 yr for the two top calibrated dates.

Lithological facies descriptions

Clay and silt sediments with shell fragments mostly filled the lagoon alternating with layers of fine sandy material. Two sedimentary units (Unit 1 and 2) and five lithological facies (A, B, C, D, and E; described below) were recognised from Core Centre, based on visual inspection (Fig. S1). Magnetic susceptibility (MS), LOI, percentage of carbonates and X-ray fluorescence (XRF) profiles were used to better define these facies. Unit 1 was identified from the base of the core at 235 cm to 81 cm depth and unit 2 from 81 cm to the core top.

Levels making up facies A are characterized by different thickness varying between 3 cm and 18.5 cm (A1 to A6), all of them belonging to unit 1. This facies is formed by gray sandy silt sediments, carbonate grains and plant fragments. Bioclasts of gastropods and bivalves, mainly *Cerastoderma glaucum* (Poiret, 1789) valves, and also foraminifera could be found.

Facies B is formed by homogeneous fine silt sediments with gray coloration (Fig. S1). Seven levels were described at this lithofacies (B1-B7) with different thickness (between 4 cm and 30 cm), all located at unit 1. They are characterized by very low sand content, plant fibre remains of variable length and microscopic carbon particles. Bioclasts of molluscs and articulated valves were usually absent or scarce (facies B2-B7).

Facies C is formed by homogeneous ochre fine silt sediments highly enriched in plant remains. Five levels were identified (C1-C5) with different thickness and present in both units 1 and 2, usually made up of narrow layers (range 3 - 36 cm). Facies C1 and C3 were composed of plant remains of different lengths, bioclasts of molluscs and articulated valves of *Cerastoderma glaucum*. Facies A5/C2 showed the typical sediment composition of facies C although highly enriched in sand. Facies C4 and C5 are composed of soft pink (C4) and orange (C5) mud sediments with carbonate grains (C4) and are confined to unit 2.

Facies D is mostly composed of homogeneous silt, with two narrow layers with some fine sand at the top. Plant and mollusc remains are very scarce.

Facies E is formed by heterogeneous dark grey silt sediments, without plant and mollusc remains. Five levels were identified as lithofacies E1 to E5, always consisting of very thin layers (range 0.5 - 3 cm). The majority of these levels coincide with MS increases (Fig. 15), mainly at E4 and E5 with the highest MS peaks. Based on the high content of detritic elements such as titanium, potassium and aluminum, this material was probably reworked and transported into the lake due to flood events.

XRF results

The high-resolution records of the most significant geochemical elements obtained by the XRF

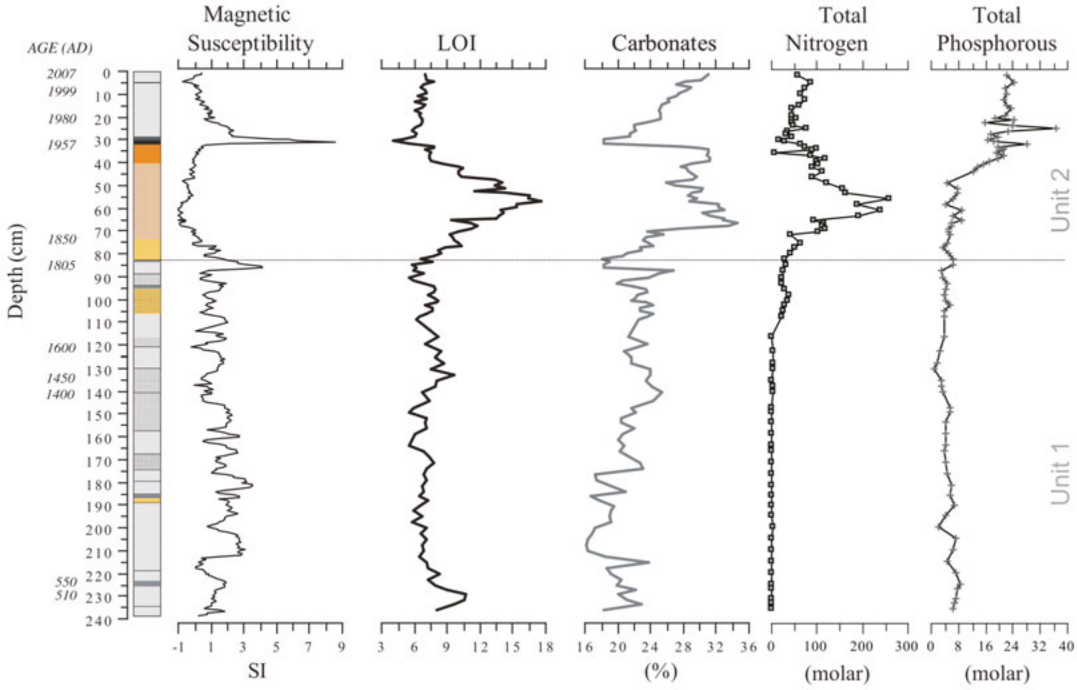


Figure 15. Magnetic susceptibility (MS), loss on ignition (LOI), % carbonates, total nitrogen (TN) and total phosphorus (TP) of Core Centre. *Susceptibilidad magnética (MS), pérdida por ignición (LOI), % de carbonatos, nitrógeno total (TN) y fósforo total (TP) del core Centre.*

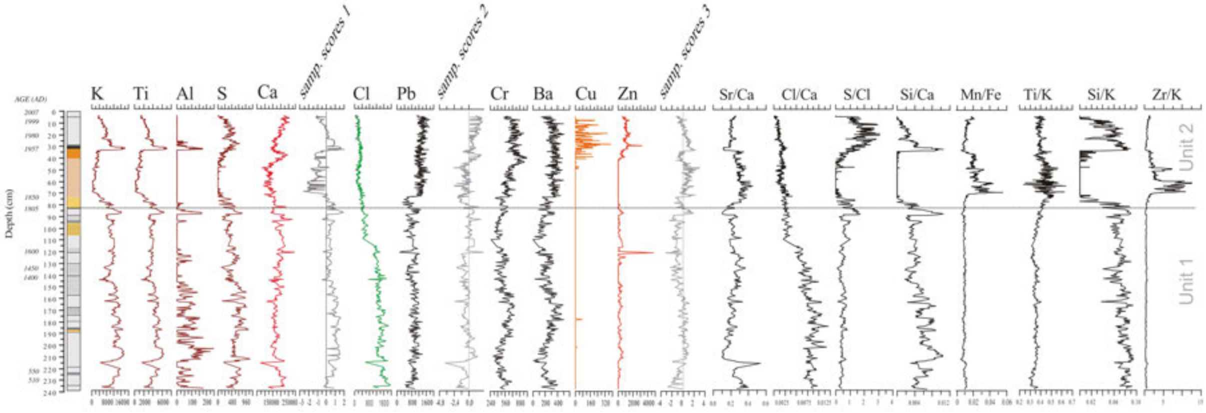


Figure 16. Stratigraphic diagram of geochemical XRF fluorescence data from Core Centre. The samples scores 1, 2 and 3 from a PCA are also plotted. See Figure S2. *Diagrama estratigráfico de los datos geoquímicos de fluorescencia XRF del core Centre. Se muestran además los valores de las muestras para los factores 1, 2 y 3 de un análisis de componentes principales (PCA). Ver Figura S2.*

analysis are plotted in figure 16. Two kinds of profiles are easily seen reflecting the distinct varying proportion of authigenic versus detrital elements with core depth. The variation of Ca and Sr can be mainly related to shell abundance (molluscs, ostracods, foraminifera, Sabatier *et al.*, 2010, 2012) (sedimentary unit 1) or to endogenic carbonate precipitation (unit 2). In the sedimentary unit 2 carbonate shells were very scarce and the increase in Ca and carbonates should be predominantly associated to precipitation of calcium carbonate due to high photosynthetic rates, first mediated by the development of aquatic macrophytes and, since the end of the 1960's (20-25 cm), by phytoplankton production, although some influence of clastic carbonate input from the watershed cannot be disregarded.

On the other hand, detrital elements, Ti, K altogether with quartz (Si) clay elements (Al, Fe, Rb), suggest allochthonous input materials (Cohen, 2003; López-Buendía *et al.*, 1999). Particularly, Ti and K are considered detrital elements from continental sources (Cohen, 2003; López-Buendía *et al.*, 1999), and are highly positively correlated with MS ($p < 0.01$) along the core, and negatively correlated with LOI and the proportion of carbonates ($p < 0.01$), suggesting that these elements were mostly deposited into the lake by inlets and particularly flood events. In addition, we find heavy elements mostly derived from human activities to increase their concentrations to the upper parts of the core; Cr and Pb increasing since about 80 cm depth and Cu and Zn later at the top of the sequence. Cl indicates the higher bottom salinity with an important shift at about 120 cm and progressively decreasing to the top of the sequence. Although this reduction in Cl content might also be related to a reduction in sediment grain size (and water accumulated therein), the salinity change is also supported by a reduction in foraminifera diversity and stenohaline marine ostracod relative abundance (see below).

PCA results (Fig. S2, available at http://www.limnetica.net/en/limnetica) confirm the opposite distribution between elements with detrital origin and characteristic of high erosive periods (or clastic input), and elements more characteristic of authigenic stable periods (or carbonate input). This is indicated by axis 1, which accounts for most of the variance of the data (90 %). Detrital elements cluster with high positive scores, Ti, Fe and K showing the higher scores followed closely by Co, Rb, Si, and to a lesser extent by S and Al. High negative scores are displayed by Ca and Sr, followed by the compact group of Ni, Cr, Pb, and by Zr and Ba. Although explaining a low proportion of the total variance, the separation along axis 2 between Zn and Cu (positive scores) and Cl and As (negative scores) reflects salinity variation. The former two elements (usually related to chemical industrial activities, but also agricultural practices in the case of Cu) are more important at the very top of the core. Due to the proximity of the lake to the sea, Cl and As (a good pyrite indicator) variation probably indicate variation in marine influence. Cl was negatively correlated with the proportion of carbonates ($p < 0.01$), suggesting that sea water played an important role in the geochemical composition of the lagoonal sediments in the lower half of the core.

The ratios between elements (Fig. 16) clearly indicate shifting paleoenvironmental conditions; Cl/Ca show the salinity change, while Sr/Ca indicates a zone of change in the type of carbonates in the lower part of unit 2 at the onset of desalinization, when macrophytes were abundant and diminished afterwards. Contrarily, the S/Cl ratio indicates a reverse pattern for S, with peaks that may indicate sulphide-rich matter originated in anoxic conditions (Zwolsman *et al.*, 1993) at the top and bottom of sedimentary unit 2, but lower values at the more stable redox conditions of macrophyte development prior to eutrophication. Also, Si as well as clay elements show a very scarce concentration in this zone, altogether with detrital elements but the reduction of the later is much less marked. Thus, the ratio Ti/K shows a slight increase also in this zone. The Mn/Fe ratio marks also clearly this zone, with Mn higher values in the stable redox (macrophyte-dominated) zone, probably due to the faster oxidation of Fe (Zwolsman *et al.*, 1993).

Foraminifera

Selected common foraminifera taxa in Core Centre are shown in figure S3. This core was (almost) barren of foraminifera in the upper 70

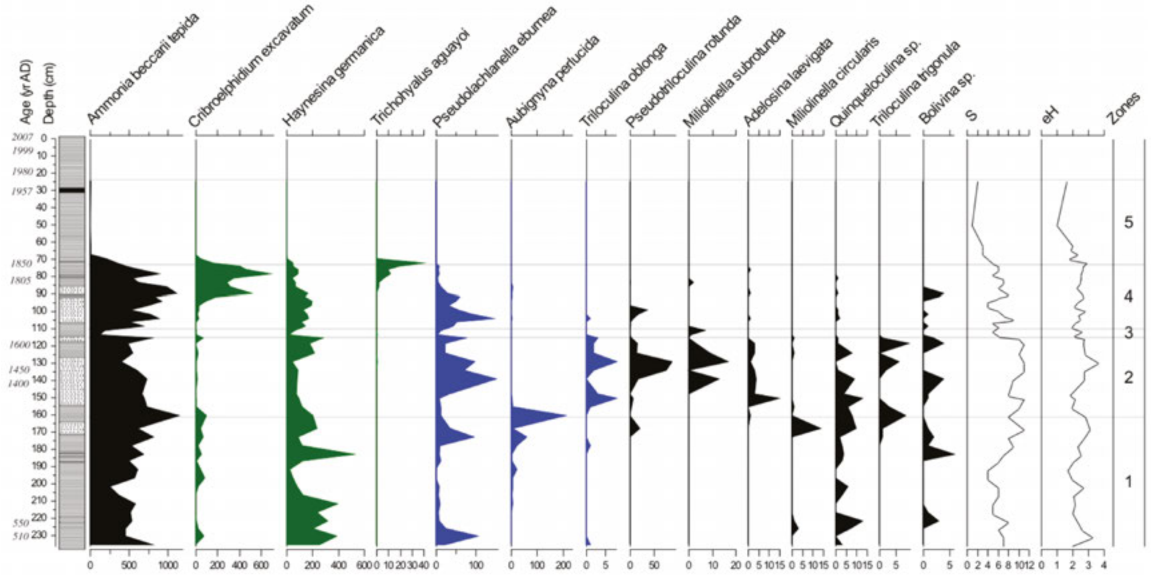


Figure 17. Summary stratigraphic diagram of foraminifera abundances in Lake Albufera core Centre showing taxa present at minimum > 5 individuals. Green: species tolerant of relatively low salinity in brackish waters (other than *Ammonia* spp.); blue: those considered less tolerant to low salinity. The diversity of order 0 (S = species richness) and order 1 (e^H, where H = Shannon diversity), and zone boundaries defined by constrained cluster analysis are shown on the right. Sediment legend as in figure 2. *Gráfico estratigráfico resumen de los foraminíferos del core Centre de la laguna de la Albufera, mostrando las especies con un mínimo de individuos > 5. Verde: especies tolerantes a salinidades relativamente bajas en aguas salobres (aparte de* Ammonia *spp.); azul: aquellas poco tolerantes a bajas salinidades. A la derecha se muestran la diversidad de orden 0 (S = riqueza de especies) y orden 1 (e^H, donde H = diversidad de Shannon), así como las zonas definidas mediante análisis constreñido de clasificación. Leyenda de la columna sedimentaria como en la figura 2.*

cm (Zone 5), with only one or two species present in some samples and at very low abundances (Fig. 17). As in the shorter core Antina, *A. tepida*, *C. excavatum* and *H. germanica* dominated the sequence. Zone 1 (235 - 161 cm) was relatively species-poor, but progressively increased in richness towards the top, attaining values close to the maxima recorded in the more diverse Zone 2 (161-114.5 cm). Zone 3 (114.5-111.5 cm) is characterized by a sharp and brief decline in abundance and diversity of foraminifera, which partly recover in Zone 4. An important peak in abundance of *C. excavatum* is observed in the top half of Zone 4, which ends abruptly at about 72 cm with the maximum abundances of *T. aguayoi*, in close similarity to the top of the foraminiferal sequence of core Antina.

Ostracod assemblages

We identified 100 952 ostracod valves, representing 18 species of ostracods from 15 genera in the Albufera Core Centre sequence. A selection of common species is shown in figure S4. Ostracods were absent from two of the 70 studied samples (at the top of the core), and 6 samples had only one species present (Fig. 18).

Cyprideis torosa together with *Loxoconcha elliptica* and *Xestoleberis nitida* were the most abundant species along the core. In addition, *Aurila arborescens*, *Candona angulata* and *Darwinula stevensoni* were also abundant, especially the first one in the lower part of the sequence. *Candona angulata* and *D. stevensoni* were abundant in the upper part and *Paralimnocythere psammophila*, *Cypridopsis vidua* and *Limnocythere innopinata* appeared with lower abundances. Other species present were very scarce.

Application of a stratigraphically-constrained cluster analysis to the ostracod assemblages allowed the differentiation of 4 main stratigraphical zones that could be further subdivided onto 7 different subzones (Fig. 18). The 4 main stratigraphic zones presented different assemblages regarding to their ecological recruitments (1: brackish; 1b: brackish-marine; 2: brackish; 3-4: freshwater).

Ecophenotypic response and isotope composition of Cyprideis torosa shells

The length of 2110 *C. torosa* valves (994 females and 1116 males) was measured from 57 subsamples (Fig. 19). LOESS regression was applied separately to female and male size changes through the core and showed that both followed the same pattern of variation in carapace length with depth through the core. Most *C. torosa* found at zones 1 and 2 showed smooth (unnoded) valves, but the percentage of *C. torosa* noded forms increased in zone 3, suggesting an important salinity decrease.

The isotope values for *C. torosa* shells ranged between -10.06 ‰ and 2.33 ‰ (mean value -5.45 ‰ ±1.61) for δ^{13}C and between -4.98 ‰ and 3.29 ‰ (mean value of -0.18 ‰ ±1.71) for δ^{18}O (Fig. 19). These two variables were not correlated for the entire sequence. δ^{18}O showed high variability among individual valves in zone 1 (235-107), suggesting a mix of waters or evaporation processes during some periods. δ^{13}C remained fairly constant along this zone. Notable variation in δ^{18}O values occurred in zone 2 (107-73 cm). δ^{13}C increased towards the mid part of this zone (but decreasing in SD) and increased again towards the upper zone boundary. The most important changes were observed for both variables in subzone 3a (73-60 cm; Fig. 19). δ18O dropped markedly towards the mid part of the subzone, reaching very low values. δ^{13}C also showed a decreasing trend, but less marked. δ^{13}C was highly variable in subzone 3b (60-39 cm), and the few isotopic shell analyses (2) performed for zone 4a show values that were somehow lower (but still in the range) as those of subzone 3b.

The isotopic results compared with previous data (Marco-Barba *et al.*, 2012b) for recent shells of *C. torosa* (Fig. S5, available at http://www.limnetica.net/en/limnetica) show that ostracod zones 1 and 2 fitted well within the range of values for typical brackish and hypersaline sites studied by Marco-Barba *et al.* (2012b). Also δ^{18}O results for zones 3 and 4 fit values corresponding to low salinity environments as compared with

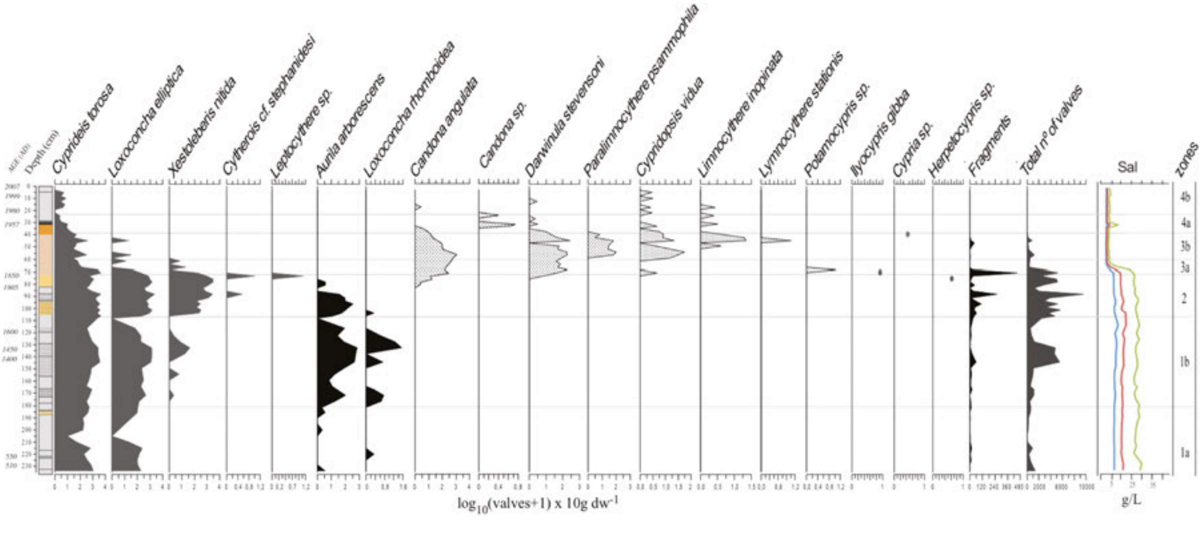


Figure 18. Stratigraphic diagram of ostracod abundances and reconstructed salinity (Sal) in Lake Albufera Core Centre. The zones defined by constrained cluster analysis are also shown. Dark grey: brackish species; black: brackish-marine species; light grey: freshwater species. *Diagrama estratigráfico de las abundancias de los ostrácodos y la salinidad estimada (Sal) en el Core Centre de la laguna de la Albufera. Se muestran además las zonas definidas por un análisis de clasificación constreñido. Gris oscuro: especies de aguas salobres; negro: especies con afinidades salobres-marinas; gris claro: especies dulceaqüícolas.*

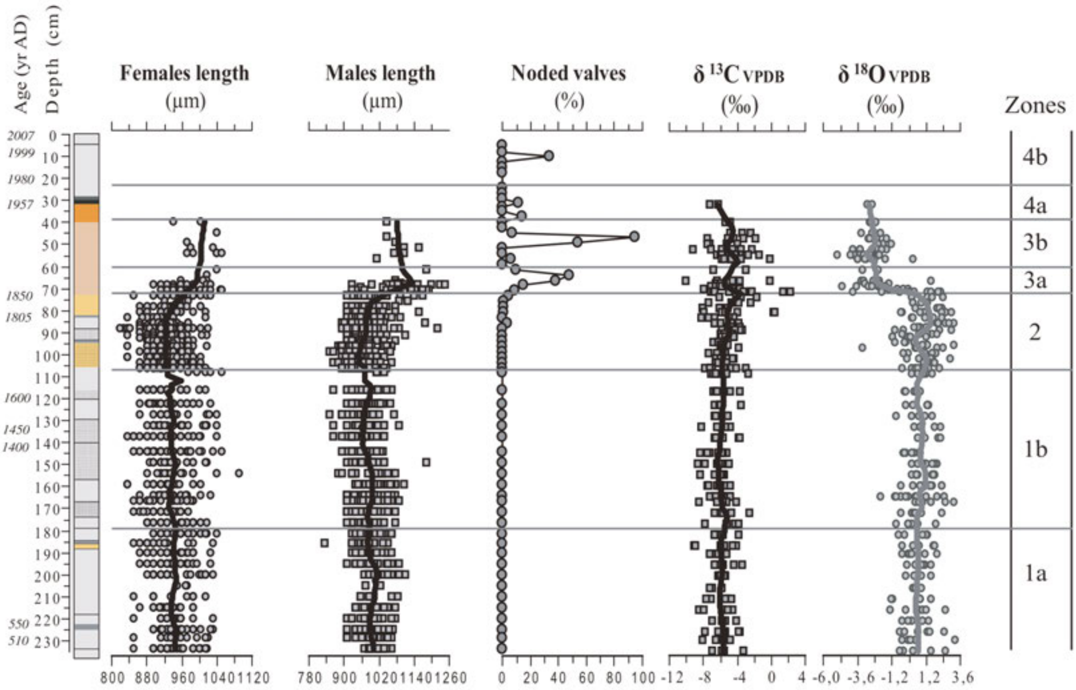


Figure 19. *Cyprideis torosa* measured carapace lengths for females and males, percentage of noded *C. torosa* valves and isotopical ratios ($^{13}C/^{12}C$ and $^{18}O/^{16}O$) performed on *C. torosa* shells along the sedimentary sequence of Core Centre. Loess regressions were performed for all ratios using average scale of 10. *Medidas de longitud de las valvas de* Cyprideis torosa *para machos y hembras, porcentaje de valvas nodadas de* C. torosa *y razones isotópicas (*$^{13}C/^{12}C$ *and* $^{18}O/^{16}O$*) de las conchas de* C. torosa *a lo largo de la secuencia sedimentaria del core Centre. Las regresiones Loess se llevaron a cabo para todas las proporciones utilizando una escala promedio de 10.*

previous geochemical data on recent ostracods obtained by Marco-Barba *et al.* (2012b) (Fig. S5). However, the data for $\delta^{13}C$ values are higher than expected given their $\delta^{18}O$ values, based on relationships defined by Marco-Barba *et al.* (2012b) in oligohaline spring ponds. This difference is expected from the different origin of water (ground- vs. surface) and by the much highest productivity of a lagoon compared to a spring pond with clear waters (Leng & Marshall, 2004).

DISCUSSION

The prehistoric Albufera lagoon (8700 – 3400 cal. BP)

Cores El Palmar and Pujol were extracted from the sand bar separating the lagoon from the sea, and their sedimentary analysis (Santisteban *et al.*, 2009) revealed the major influence of marine transgression and regression events on its depositional history. As reported previously for El Palmar (Marco-Barba *et al.*, 2013a), both sequences are dominated by brackish water ostracod taxa (euryhaline and brackish stenohaline) rather than fully marine, species-rich (e.g. Ruiz *et al.*, 2000) assemblages, indicating that the area was not widely open to the sea during most of the Holocene (Fig. 20). The analysis of foraminifera and diatoms confirm the interpretation, as indicated by the dominance of *A. beccarii* and associated forams, and (where preserved) *Mastogloia* taxa and other obligate saline brackish diatoms. The exception is in the lower Pujol sequence, where peaks in foraminiferal diversity occur during a phase thought to represent a sandy beach in a transgressive period (Santisteban *et al.*, 2009), suggesting stronger marine influence in the northern part of the recent barrier, which is at present much closer to the seashore than Palmar.

For the same period, Carmona *et al.* (2016) also found a brackish lagoon foraminiferal community in the northern part of the Albufera wetland, in a site location occupied by ricefields since the early 19th century.

At about 8300 cal. BP, there is a change in the type of sediments (muddy to sandy), and a sharp reduction event in ostracod, foraminiferal and algal remains abundance, coincident with an important peak in megaspores (Fig. 20). Modern megaspores are produced by heterosporous lycopods and ferns (Traverse, 2007), and are considered a proxy for the presence of tall grasses and rushes common on the borders of freshwater and brackish wetlands (Goodman *et al.*, 2008). Therefore, a relative increase may indicate a reduction in the inundated part of the lake in relation to the littoral area. If the chronology is accurate, this event might be related to the 8.2 event (Bond *et al.*, 1997), which could have triggered a reduction of the water table in the wetland at that time. Also, Carmona *et al.* (2016) relate the base of a sequence from the northwestern part of the lake, which consisted of a peat layer, to a potentially cold and dry period. This period is brief, and ostracod and foraminiferal communities rapidly recover. Subsequently, between about 7500 and 7100 cal. BP, another longer lasted and major reduction

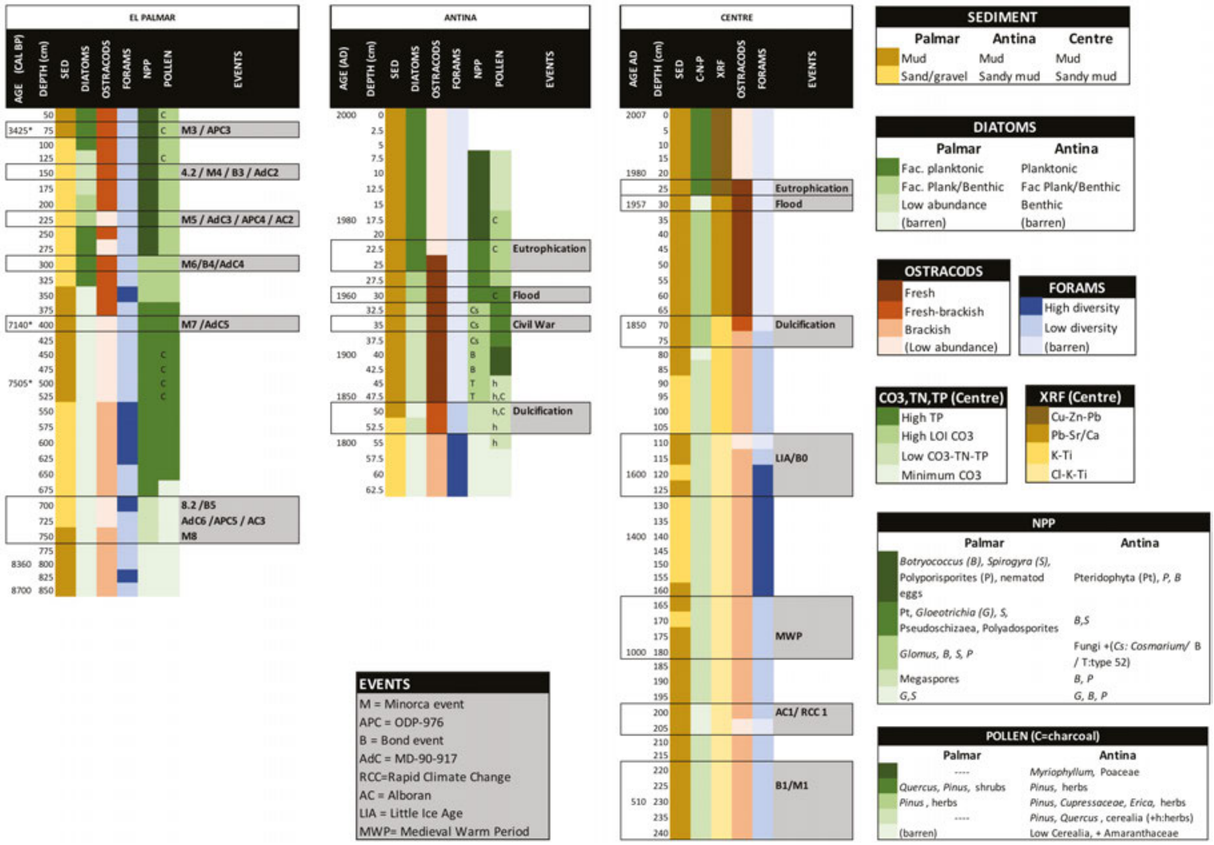


Figure 20. Summary of main changes detected for different proxies in the Albufera de València sequences of El Palmar, Antina and Centre. The chronological location of main events are to be taken as approximate, particularly for Core Palmar, considering the low resolution of the chronology. * ^{14}C cal. dates; other dates are estimated from the chronological models. See text for further explanations and for more detailed proxy sequences and interpretations. *Resumen de los principales cambios detectados para diferentes indicadores de las secuencias de El Palmar, Antina y Centre de la Albufera de València. La localización cronológica de los principales eventos debe considerarse aproximada, particularmente para el core Palmar, teniendo en cuenta la baja resolución de la cronología. * dataciones calibradas* ^{14}*C; otras fechas son estimaciones a partir de los modelos cronológicos. Véase el texto para más explicaciones y mayores detalles de las secuencias de los indicadores y su interpretación.*

in ostracod abundance is recorded, coinciding with a shift in sediment composition, in foraminiferal composition and a reduction in species richness, and the common presence of soil fungal remains (Fig. 8, Fig. 20). This phase also suggests an aridity period, that might correspond to the M7 Minorca event (Frigola *et al.*, 2007), although it could also relate to an inland advance of the sand barrier (aggradation) at the point of core extraction (Santisteban *et al.*, 2009), reducing the lagoon to a series of (temporary) shallow interdune ponds (although no ostracod remains typical of temporary water bodies such as those described by Rueda *et al.* 2006 were observed).

Above this part of the sequence, proxy data indicate environmental changes that would create a wetland and landscape quite different to its previous state. Indeed the largest palaeoenvironmental change through the El Palmar core is observed at about 4 m depth (7140 cal. BP) (Fig. 20), when ostracods, diatoms and other algal remains (e.g. *Botryococcus*, *Gloeotrichia*, *Spirogyra*) start to show a more stable oligohaline (see e.g. Margalef, 1983) water body from here on. Diatoms are preserved for the first time at 312 cm depth, indicative of a shallow, brackish, environment. The salinity optimum of *P. brevistriata* is quite high because it will tolerate brackish water but it also occurs in fresh water, as do all of the facultative planktonic taxa encountered. The maximum abundance of these taxa in zone 1 (341.5-251.5 cm) could indicate lower salinity but is difficult to interpret. Shifts between facultative planktonic and benthic diatom taxa could also result from changes in water depth, but (in the absence of true plankton) are more likely to indicate changes in the proportion of reeds (FP) to mud/stone habitat. Ostracod palaeoassemblages and ecomorphogenetic changes provide more definitive evidence for lower salinity at the top of the sequence (Marco-Barba *et al.*, 2013a), but always in the oligohaline-mesosaline range. The reasons for intermittent diatom preservation are not clear; since there is no evidence for hypersalinity in samples containing sparse assemblages, it is more likely to be a function of a high energy environment (breakage and poor burial) in the turbid, shallow water of the littoral zone; the complete absence of diatoms at the base of the sequence may be a function of low productivity prior to erosion of nutrients into the lake with deforestation (as suggested by pollen remains – see below), or could reflect more rapid burial with enhanced sediment input.

But not only major changes in the functioning of the water body are found, as also we observe major shifts in landscape vegetation, as suggested by palynological data. Prior to 7140 cal. BP, the vegetation was dominated by trees (*Quercus*) and bushes (*Erica*, *Cistus*), but above the layer that might indicate the M7 event (Frigola *et al.*, 2007), Poaceae (including Cerealia-type) dominate, suggesting a major climate change effect towards higher aridity, although this contrasts with the western Mediterranean increased precipitation at about 6000 BP suggested by general models (Roberts *et al.*, 2011), so as with the reduction in salt content in the lake as suggested by microfossils. Vegetation changes coincide with a change in the depositional environment (lagoonal to sandy lobes), so that the palynological change observed might also be related to the local development of grasses in these new sandy environments. Further decreases in arboreal pollen through the top half of the sequence are also coeval with known aridity events (M3-M6; Frigola *et al.*, 2007).

We cannot however rule out the potential influence of human impact on landscape changes at these early times. A hint to these effects is the frequency above 350 cm of egg cover remains of parasitic worms (Nematoda), probably related to increased cattle raising activities around the lake. Fire events (charcoal frequency) had already increased prior to deforestation, but charcoal remains subsequently increased in frequency in the top half of the sequence. Human populations are known to have occupied the territory near the lake (a few km inland and tens of km southward) and had used its resources (e.g. cockles, grain) at least since about 6000 BP (Mateu *et al.*, 1985; Martí-Oliver, 1988). Not too far, at about 50-70 km to the south, major vegetation changes during this period have been suggested to be human-induced through fires (Carrión & van Geel, 1999; Carrión *et al.*, 2010), and increased livestock activities at around 6200 cal. BP have been associated to forest decline near Neolithic settlements (Dupré, 1995; Badal *et al.*, 2012).

The historic Albufera lake (500 AD- 2007)

Unfortunately, according to the chronology of our cores, we have no data for palaeoenvironmental change between 3400 and 1500 cal. BP in the Albufera lagoon. However, a sequence analysed by Carmona *et al.* (2016) from the northwestern part of the wetland includes this period. These authors found no major difference in foraminiferal species composition from the oldest sediments, since c. 8200 up to 1000 cal BP, being dominated by *A. tepida*, *H. germanica* and *C. excavatum*, indicating a brackish coastal lagoon. This association is very similar to that found dominating the base of our shortest core Antina and most of the longer Core Centre.

The bottom half of Core Centre shows in general homogeneous traits in many proxies (Fig. 20), including elements detected by XRF, magnetic susceptibility, LOI, N and P content, ostracod geochemistry and shell size of *C. torosa*, so as in dominant species of ostracods (*C. torosa*, *L. elliptica*) and foraminifera (*A. tepida*, *H. germanica*). Still, some striking events can be observed and interpreted. First, there is an unusually rapid shift in various elements that decrease at 210-215 cm and increase to high values at 200-205 cm (an approximate estimated age of 700-800 AD) (Fig. 16). These include minimum values (and subsequent peaks) of K, Ti, Al, S, Ca, Cl, Si/Ca and inverted peaks in Sr/Ca. At the same point, there is a minor peak in magnetic susceptibility and minimum values in carbonate content. Also, the stratigraphy shows a remarkable absence of macroremains and a sudden drop in ostracod and foraminiferan abundance (Fig. 20). A potential explanation could be a flood event (increased sediment input and reduced density of shell remains) or some sort of rapid climate change. At this time, Moberg *et al.* (2005) found a peak in global temperature after a cold period. The established arid periods AC1 (Alborán events; Cacho *et al.*, 2001) and RCC1 (Rapid Climatic Change periods; Mayewski *et al.*, 2004) might also be responsible for such a rapid event. Unfortunately, we have no pollen data from this core to see if vegetation suffered some change at this time to validate this hypothesis. Another change in the lower sequence, from 180-160 cm (i.e. 1000-1200 yr AD), occurs when brackish (and some marine) ostracods and foraminifera increase progressively in abundance and diversity indicating more stable conditions in a brackish lagoon, at the time of the Medieval Warm Period. Later on, at about 1700 AD (110 cm depth), another sudden drop in ostracod and foraminifer abundance and diversity occur, probably related to reduced productivity at low temperatures during the Little Ice Age (although our chronological model has to be taken with caution).

The most important change through the last 1500 years in the lake record is observed at a depth of 70-75 cm in the Core Centre, and 50-55 cm in core Antina (Marco-Barba *et al.*, 2013b for ostracods, and this survey for other remains), both corresponding to the beginning of the 19th century. Here, the system changes completely from a brackish to an oligohaline lagoon. Diatoms and cladocerans (Marco-Barba *et al.*, 2012a) start to be common, while foraminifera disappear, at the same time that the ostracod palaeocommunity shifts from one dominated by unnoded, small, *C. torosa* and accompanying brackish species to one with noded and larger *C. torosa* and typical freshwater dwellers. Ostracod shell $\delta^{18}O$ drops and Sr/Ca values increase (Marco-Barba *et al.*, 2013b). Altogether this indicates a reduction in salinity from about 15 to less than 2 g/L (Marco-Barba *et al.*, 2013b). K, Ti, Al and S drop, and Pb, Cr and Ba increase, together with LOI, carbonates and N content. An initial increase in S/Cl may suggest increased sulphide (Zwolsman *et al.*, 1993) at the start of the freshening phase, probably indicating biomass decomposition at the collapse of the brackish adapted communities. The most intense human impact occurred during this period. The Spanish crisis at the beginning of the XIX century and the changes in the property of the surrounding land leaded to intensive agricultural expansion and to strong hydrological control of the Albufera de Valencia (Sanchis Ibor, 2001; Marco-Barba *et al.*, 2012; 2013b). The lagoon area was reduced in 6000 Ha and became hydrologically controlled for rice farming (Mateu, 1987; Sanchis Ibor, 2001). This increase in rice cultivated area may correspond to the pollen local signal of increased Cerealia-type remains in the Antina core, co-occurring with the reduction in Amaranthaceae, indicators of more saline environments.

As a response to this drastic salinity change, the macrophyte community also switched from dominance by salt tolerant species such as *Lamprothamnium papulosum* (Wallroth) or *Chara aspera* Detharding ex Willdenow to freshwater species including *C. vulgaris* L., *C. tomentosa* L. and *C. hispida* L., as shown by previous research (Rodrigo *et al.*, 2010; Rodrigo & Alonso-Guillén, 2013), and including *Myriophyllum* sp., as indicated by the peak in our pollen data. The core depth of this freshening and the age model (based on ^{210}Pb-^{137}Cs dating) provided by Rodrigo *et al.* (2010) fit very closely to our results. In contrast, Carmona *et al.* (2016) found that freshening took place much earlier, at about 820 cal. BP according to their ^{14}C dates. Notwithstanding potential chronological uncertainties, a possible explanation of these large differences might come from wetland spatial heterogeneity, as the site in Carmona *et al.* (2016) is much further inland and close to a ravine, which could have promoted the change to an oligohaline wetland in that area during wettest periods and/or with reduced flow connections with the main lagoon water body (i.e. it might be flowing only downstream to the central lake but not backwards, unless the site was fully open to the lake and wind could easily mix the waters).

At the beginning of the 20th century, the palaeolimnological data and geochemistry show no major changes (other than increased LOI and N content, related to the development of a macrophyte meadow and increased trophic state; Marco-Barba *et al.*, 2012a) but the pollen and NPP remains start to change, indicating a certain recovery of forested (*Pinus* spp.) areas, partially interrupted shortly during a time corresponding approximately to the Spanish Civil War (1936-1939). At the point when expanding forests retreat again (maybe because of logging for winter heating or cooking), an increase in Cyperaceae and Poaceae occurs, perhaps related to more intense use of wet areas surrounding the lagoon for cattle raising (*Plantago* in zone B1, Fig. 12, and Polyadosporites in zone A2, Fig. 13; López-Sáez and López-García, 2003), a longstanding practice since at least the late 13th century (Rosselló i Verger, 1995) which is known to have continued during the 16th century (Sanchis Ibor, 2001), though perhaps reduced after ricefield expansion.

At the end of the first half of the 20th century, despite the huge ecosystem change, the water quality of the lake was still high, with a well-developed macrophyte community and rich in benthic and planktonic groups indicative of clear waters (Pardo, 1942; Fundació d'Amics del Parc Natural de L'Albufera, 2000). This is in agreement with the observed peak in chlorophytes with a preference for oligotrophic waters such as *Cosmarium* (see Margalef, 1983) in the Antina core. However, nutrient input to the lake continued to rise, particularly P, and *Cosmarium* started to be replaced by more tolerant groups such as *Botryococcus* or *Spirogyra* (Margalef, 1983).

By the end of the 60's and during the early 70's, an increase in human population, urban development and industrialisation, would definitely drive another major change in the lake towards its present hypereutrophic and polluted state, indicated by the diatom *Cyclotella meneghiniana*, for example. The lagoon was heavily artificially modified by this time. The previous rice field expansion had already caused a reduction of the area of the lake, with extensive use of fertilizers. Fine mud sediments, the absence of mollusc shells and macrophyte remains, reduced ostracod density and a major change in diatom composition favouring planktonic species, characterized this period. The reduction in benthic organisms (ostracods and diatoms) was driven by poor water quality, the loss of macrophytes and the anoxic conditions at the bottom sediments (Marco-Barba *et al.*, 2012a), coupled with increased productivity. The uncontrolled watersheds, agricultural residues and industrial spills into the lake modified the natural conditions of the system driving it to an hypereutrophic state. The collapse of macrophytes in a hypereutrophic system coincides with a prolonged peak in sediment sulphide (as S/Cl), added to pollution by heavy metals (Pb, Cr, Zn) and, consequently, to low water quality and the loss of biodiversity (Vicente & Miracle, 1992; Romo *et al.*, 2005; Poquet *et al.*, 2008; Marco-Barba *et al.*, 2013b). Hopefully, the effects of sewage diversion on reducing nutrient load into the lake (Soria, 2006) might drive a future drop in the trophic conditions of Albufera and perhaps some recovery of the previously diverse benthic community.

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Dystrophication of lake Suchar IV (NE Poland): an alternative way of lake development

Edyta Zawisza[1,*], Izabela Zawiska[2], Krystyna Szeroczyńska[1], Alexander Correa-Metrio[3], Joanna Mirosław-Grabowska[1], Milena Obremska[1], Monika Rzodkiewicz[4], Michał Słowiński[2] and Michał Woszczyk[4]

[1] Institute of Geological Sciences, Polish Academy of Sciences, Research Centre in Warsaw, ING PAN, Twarda 51/55, PL-00818 Warsaw, Poland.
[2] Institute of Geography and Spatial Organization, Polish Academy of Sciences, Twarda 51/55, PL-00818 Warsaw, Poland.
[3] Instituto de Geología, Universidad Nacional Autónoma de México, Ciudad Universitaria, 04510 CDMX, Mexico.
[4] Institute Geoecology and Geoinformation, Adam Mickiewicz University, Bogumila Krygowskiego 10, PL-61680 Poznań, Poland.

* Corresponding author: ezawisza@twarda.pan.pl

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ABSTRACT

Dystrophication of lake Suchar IV (NE Poland): an alternative way of lake development

The long-term dynamics of a lake development and the role of the catchment and climate change in the dystrophication process were studied. Lacustrine sediments from Lake Suchar IV, NE Poland (880 cm long core), were selected for paleolimnological studies. The paleoecological reconstruction was based on pollen, subfossil cladocerans, diatoms, macrofossils and chemical analyses. Sediment chronology was based on the results of ^{14}C dating and palynological analysis. The obtained data showed that the lacustrine sedimentation at Lake Suchar IV began in the Late Glacial and continues to this day. The plant succession indicates that sedimentation processes started as early as the Oldest Dryas ~ over 15 000 cal yr BP ago. Important changes in the pollen spectrum of aquatic plants were noted from the mid-Atlantic period. At that time, communities of *Nymphaea* and most species of green algae disappeared from the lake, which indicates a significant ecological transformation in the aquatic environment. This transformation is also manifested in the subfossil Cladocera and diatom communities. The Cladocera community in the sediments of Lake Suchar IV was represented by 37 species, mostly littoral ones. From the transition time (7200–5600 cal yr BP), Cladocera communities were dominated by taxa tolerant of acidification such as *Alonella excisa*, *Acroperus harpae*, *Alona affinis* and *Alonella nana*. A total of 193 diatom taxa (species and varieties) were identified in the sediment, including 172 belonging to *Pennales* and 21 to *Centrales*. In general, the diatom community was dominated by alkaliphilous – on average 45 % of the species composition. In terms of trophic preferences, the largest group of diatoms was represented by oligotraphentic, meso-eutraphentic and eutraphentic taxa. No diatoms were present during about 6000 cal yr BP from the transition stage. Sediment geochemistry and macrofossils also indicate the limnological development and climate trends.

The obtained paleolimnological results indicate that Lake Suchar IV has undergone an ecological transformation from a harmonic to disharmonious ecosystem. During the Late Glacial and the early Holocene, Lake Suchar IV was a typical harmonic lake that transformed into a dystrophic state at the end of the Atlantic period. Sediments of Lake Suchar IV also show a very interesting sequence of trophic changes, from oligotrophic to mesotrophic and to dystrophic conditions.

Key words: Suchar IV, Lake history, Dystrophy, Harmonic/disharmonic state, Paleolimnology studies, Wigry National Park, NE Poland

RESUMEN

Distrofización del lago Suchar IV (NE Polonia): Un camino alternativo del desarrollo de un lago

Se estudiaron la dinámica a largo plazo del desarrollo del lago y el papel de la cuenca de captación y el cambio climático en el proceso de distrofia. Se realizó un análisis paleolimnológico de los sedimentos del lago Suchar IV, NE Polonia (core de 880 cm de largo). La reconstrucción paleoecológica se basó en el análisis del polen, los cladóceros subfósiles, las diatomeas, los macrofósiles y el análisis químico. La cronología de los sedimentos se basó en los resultados de la datación de ^{14}C *y el análisis palinológico. Los datos obtenidos mostraron que la sedimentación lacustre en el lago Suchar IV comenzó en el período Glacial tardío y continúa hasta ahora. La sucesión de plantas indica que los procesos de sedimentación comenzaron hace 15 000 cal años AP en el periodo Dryas primitivo. Se observaron cambios importantes en el espectro del polen de las plantas acuáticas a partir del período Atlántico Medio. En ese momento, las comunidades de* Nymphaea *y la mayoría de las especies de algas verdes desaparecieron del lago, lo que indica una transformación ecológica significativa en el medio acuático. Esta transformación también se manifiesta en las comunidades de cladóceros subfósiles y en las diatomeas. La comunidad de cladóceros en los sedimentos del lago Suchar IV estuvo representada por 37 especies, en su mayoría litorales. A partir del tiempo de transición (7200-5600 años cal BP), las comunidades de cladóceros estuvieron dominadas por taxones tolerantes a la acidificación como* Alonella excisa*,* Acroperus harpae*,* Alona affinis *y* Alonella nana*. Se identificaron un total de 193 taxones de diatomeas (especies y variedades) en el sedimento, incluyendo 172 pertenecientes a Pennales y 21 a Centrales. En general, la comunidad de diatomeas estaba dominada por especies alcalófilas, en promedio el 45 % de la composición de las especies. En términos de preferencias tróficas, el grupo más abundante de diatomeas estaba representado por taxones* oligotrafénticos, meso-eutrafénticos *y* eutrafénticos. *No se encontraron diatomeas durante 6000 años cal BP desde la etapa de transición. La geoquímica de los sedimentos y los macrofósiles también indican el desarrollo limnológico y las tendencias climáticas.*

Los resultados paleolimnológicos obtenidos indican que el lago Suchar IV ha sufrido una transformación ecológica de un ecosistema armónico a uno disarmónico. Durante el Glaciar Tardío y el Holoceno temprano, el lago Suchar IV era un lago armónico típico que se transformó en un estado distrófico al final del período Atlántico. Los sedimentos del lago Suchar IV también muestran una secuencia muy interesante de cambios tróficos, desde condiciones oligotróficas a mesotróficas y distróficas.

Palabras clave: *Suchar IV, historia lacustre, distrofia, estado armónico y disarmónico, estudios paleolimnológicos, Parque Nacional Wigry, NE Polonia*

INTRODUCTION

At the middle and upper attitudes of the northern hemisphere (North America, Europe, Asia), a large number of lakes can be found in areas that were covered by ice during the Last Glaciation (Vistulian, Würm, Wisconsin). Over time, from the deglaciation to modern times, these lakes have witnessed environmental and climatic changes (Smol, 1992; Kernan *et al.*, 2009). Thus, sediments that are progressively deposited in lakes by diverse agents are a natural archive of the changes occurring at regional and local geographic scales (Smol, 2002; Rosen *et al.*, 2009). Given continuous sediment deposition through time, the composition of lake sediments reflects the regional climatic history, and also more local factors such as human activities (Battarbe & Benion, 2012). In areas where human activities have been absent or of limited extent (e.g. Scandinavia, Poland, Russia, Canada), lake sediments reflect an environmental history that can be almost entirely attributable to natural variability (Bos & Cumming, 2003; Luoto *et al.*, 2012; Korosi & Smol, 2012; Zawisza *et al.*, 2016). In NE Poland, a region characterized by low human population densities, lake sediments represent archives of the natural climatic and ecological conditions that have prevailed through the last several thousand years. Numerous paleolimnological studies from the region (e.g. lakes: Wigry (Zawisza & Szeroczyńska, 2007), Linówek (Gałka *et al.*, 2014), Romoty (Mirosław-Grabowska *et al.*, 2015), Suchar II (Drzymulska *et al.*, 2014), Czarne Lake (Karpińska – Kolaczek *et al.*, 2016) and from Central and Northern Europe e.g. Arapisto (Luoto *et al.*, 2012); Abisko (Bigler *et al.*, 2002); Tsulbmajarvi (Seppä & Wechström, 1999) have shown that lakes have mostly developed under natural climatic regimes, and only the youngest sediments (from the Middle Ages or industrial revolution) have been affected by human activities (Korsman *et al.*, 1994; Ott *et al.*, 2017).

Paleolimnological reconstructions of the lakes that formed during the Last Glaciation in the area of N Poland usually shows typical harmonic development (Kajak, 1998; Kupryjanowicz, 2007; Zawisza & Szeroczyńska, 2007), represented by one of the following steps of the lake successions: (1) from oligotrophy through mesotrophy to eutrophy and encroachment of vegetation or transformation to peat bogs; (2) from oligotrophy to dystrophy; and (3) from oligotrophy to a lake overgrown with vegetation. These harmonic changes are reflected in the composition of flora and fauna and in the chemical composition of lake deposits, reflecting ecological and climatic changes that occurred during the Late Glacial and the Holocene. Contrary to harmonic lakes, disharmonic lakes (e.g. lobelia and polyhumic lakes) are oligotrophic from their origin, with high limitation of Ca^{+2} ions, and are later transformed into polyhumic and/or dystrophic due to high allochtonous organic matter input (Sobek *et al.*, 2007; Rosen *et al.*, 2009; Weyhenmeyer & Karlsson, 2009). Although the definition of dystrophy is still unclear and the process of dystrophication is not well understood, paleolimnological studies of these lakes enable us to follow and reconstruct the natural dystrophication process. Very low primary production based on the allochthonous matter is a characteristic feature of dystrophic (polyhumic) lakes, with the water characterized by high contents of Total Organic Carbon (TOC), which distinguishes them from oligotrophic lakes (Hessen, 1992; Kulberg *et al.*, 1993; Porcal *et al.*, 2009).

Dystrophic lakes in the Central European Lowlands are usually considered natural relicts of past environmental conditions. Therefore, these humic lakes are believed to have undergone minor changes over the last thousand years (Górniak, 1996; Drzymuska *et al.*, 2013). Indeed, because of their extraordinary nature, dystrophic lakes are protected by national and international laws (e.g. EU Habitat Directive) as National Parks, Nature Reserves, or Natura 2000 sites. Nevertheless, some studies have demonstrated

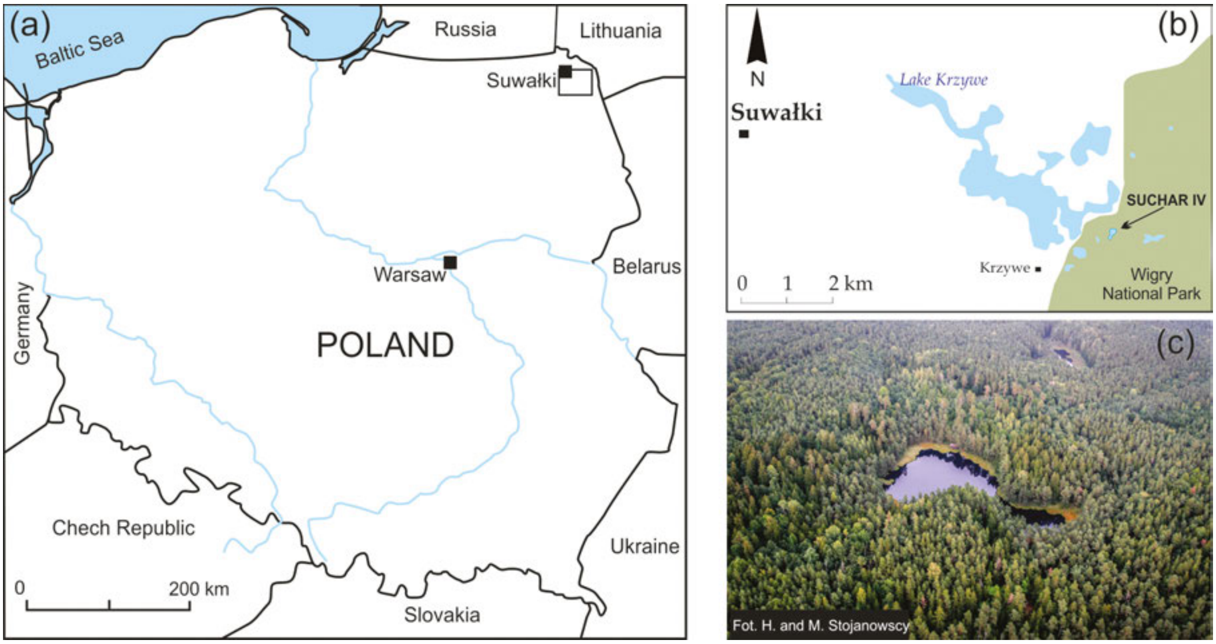


Figure 1. Location map of Lake Suchar IV: (a) location in Poland; (b) location of Lake Suchar IV in Suwalki region, green colour on the map indicated area of Wigry National Park; (c) photography of Lake Suchar IV surrounded by *Pinus* forest (photo by www.sudawcy.blogspot.com). *Mapa de la localización del Lago Suchar IV: (a) localización en Polonia; (b) Localización del lago Suchar IV en la región de Suwalki; el color verde en el mapa indica el Parque Nacional Wigry; (c) fotografía del lago Suchar IV rodeado por un bosque de* Pinus *(fotografía por www.sudawcy.blogspot.com).*

that even dystrophic lakes can shift from dystrophy to eutrophy because of anthropogenic nutrients input (Zawiska *et al.*, 2013; Drzymulska *et al.*, 2014; Rantala *et al.*, 2015). Regional climate changes derived from global human activities represent an additional threat to these unique ecosystems (Curtis, 1998).

The main objective of this study was to determine whether dystrophic lakes are hyper-stable ecosystems. We use a continuous sediment sequence from Lake Suchar IV (NE Poland) covering from the Oldest Dryas to present to study the stability of the lake, aiming to find out if its dystrophic state is derived from climate changes during the Late Glacial and the Holocene. Thus, our study sheds light on the nature of the dystrophication process reconstructing at the same time the changes experience by the local and regional flora and fauna. A natural byproduct of this study is therefore the description of the typical flora and fauna association of dystrophic environments.

MATERIAL AND METHODS

Study site

Lake Suchar IV is located in NE Poland, in the area of Wigry National Park (Fig. 1), at an altitude of 155 m a.s.l. Wigry National Park is situated in the range of the Pomeranian Phase of

Table 1. ^{14}C dates from Lake Suchar IV sediments, bls - below lake surface. Age years cal BP determined by program online CalPal (http://www.calpal-online.de). *Fechas ^{14}C procedentes de los sedimentos del lago Sucher IV, bls – por debajo de la superficie del lago. Edad en años* cal BP *determinados con el programa* CalPal *(http://www.calpal-online.de).*

samples number	depth (cm bls)	type of dating	laboratory number	^{14}C yr BP	age cal yr BP
1	860-865	conv.	MKL-1190	3250 ± 80	3493 ± 85
2	947.5-952.5	conv.	MKL-1191	3405 ± 70	3772 ± 98
3	1145-1050	conv.	MKL-1192	4750 ± 70	5767 ± 99
4	1160-1165	conv.	MKL-1193	5370 ± 85	6046 ± 109
5	1275-1280	conv.	MKL-1194	7920 ± 70	8795 ± 134
6	1335-1340	conv.	MKL-1196	8890 ± 90	9976 ± 161
7	1380-1385	conv.	MKL-1197	8980 ± 110	10065 ± 154
8	1425-1430	conv.	MKL-1201	9480 ± 100	10826 ± 199
9	1470-1475	conv.	MKL-1202	10820 ± 110	12213 ± 108
10	1535	AMS	Poz-46264	12150 ± 80	13526 ± 245
11	1545	AMS	Poz-46268	12150 ± 60	13788 ± 236
12	1560	AMS	Poz-46265	11880 ± 70	13998 ± 150
13	1620	AMS	Poz-46267	12580 ± 70	14928 ± 301

the Last Glaciation – Weichselian (Marks, 2002), within the Lithuanian Lakeland region (Kondracki, 1994). Within the Polish lowlands, the NE region represents the most severe climatic regime with a mean annual air temperature and precipitation of 5.3 °C and 593 mm, respectively (Grabowska-Bajkiewicz, 1997). The winter lasts about four and a half months, with a mean temperature ranging from -6.7 to -2.7 °C, and the ice cover lasting approximately 3 months, from the end of December until the end of March. The vegetation resembles the boreal zone and is characterized by coniferous forests with a growing season of approximately 190 days per year (Grabowska-Bajkiewicz, 1997).

With a kettle shape, Lake Suchar IV is a small water body (approximate area of 0.95 ha and z_{max} of 8.0 m) of glacial origin (Górniak, 2006). With a dystrophy index (HDI) of 104.2 (Górniak, 2006), one of the highest for the Polish area, Lake Suchar IV represents a clear example of dystrophic lake. The water pH is around 4.6, conductivity is 27 µS/cm, and dissolved oxygen is 12.0 mg/L. The entire catchment of the lake is occupied by coniferous forests. The shoreline is overgrown with floating vegetation mats, composed mostly of *Vaccinio uliginosi-Pinetum* and *Sphagno girgensohnii-Piceetum* (Górniak, 2006; Zawiska *et al.*, 2013).

Sampling

In summer 2010, an 855-cm-long sediment core was retrieved from Lake Suchar IV, using a Livingstone-type corer from the deepest part of the lake (54° 05’ 23” N 23° 01’ 30” E, water depth ~8.0 m). After coring, the core was packed and transported to the laboratory for a lithological description and sediment subsampling at 5-to-10-cm intervals. Subsamples were taken for pollen, subfossil cladocerans, diatoms, macrofossils and basic geochemistry.

Chronology

The chronology was constructed using thirteen ^{14}C dates distributed along the core. Whereas nine dates were based on bulk sediment analyzed by ^{14}C conventional method at the Laboratory of Absolute Dating in Cianowice, the other four were based on terrestrial plant macrofossils analyzed though ^{14}C AMS at Poznań Radiocarbon Laboratory (Table 1). ^{14}C dates were calibrated using the curve IntCal13 (Reimer *et al.*, 2013) and selected calibrated age-depth data pairs were used to fit a Bayesian age-depth model (Fig. 2) though Bacon (Blaauw & Christen, 2011). An alternative chronological model was built incorporating the appearance depth of biostratigraphic markers of the Central European chronostratigraphy in the sediments of Lake Suchar IV (Mangerud *et al.*, 1974; Walanus & Nalepka, 2010).

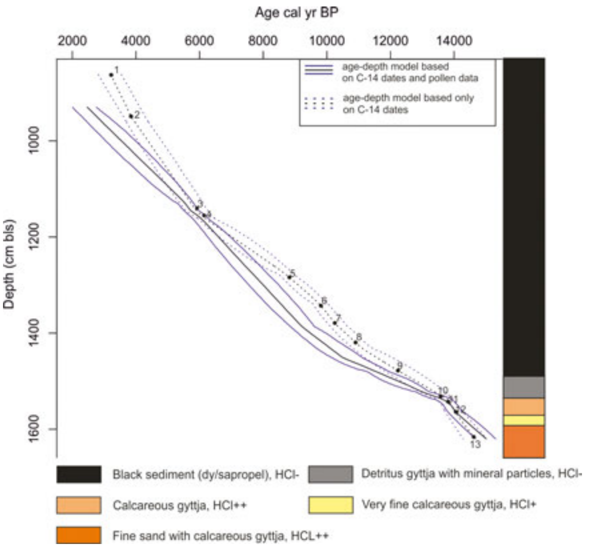


Figure 2. Age-depth model based on the ^{14}C dating of Lake Suchar IV sediments, showing sediment accumulation from Late Glacial to current times. Black dots (from 1 to 13) on diagram indicates ^{14}C date (see Table 1). Dashed lines represent age-depth model based only on ^{14}C dates and solid lines represent age-depth model based on ^{14}C dates and chronostratigraphical (pollen) borders according to Mangerud *et al.* (1974) and Walanus & Nalepka (2010). Right column is shows schematic lithology of sediment profile of Lake Suchar IV. *Modelo de edad-profundidad de los sedimentos del lago Suchar IV basado en la datación ^{14}C, indicando la acumulación de sedimentos desde el Glacial tardío hasta la actualidad. Los puntos negros (desde 1 hasta 13) en el diagrama, indican la fecha ^{14}C (ver tabla 1). La línea discontinua representa el modelo edad-profundidad basado únicamente en fechas ^{14}C, y la línea continua representa el modelo edad-profundidad basado en fechas ^{14}C y fronteras cronoestratigráficas de acuerdo con Mangerud* et al. *(1974) y Walanus & Nalepka (2010). La columna derecha muestra la litología esquemática del perfil de sedimento del lago Suchar IV.*

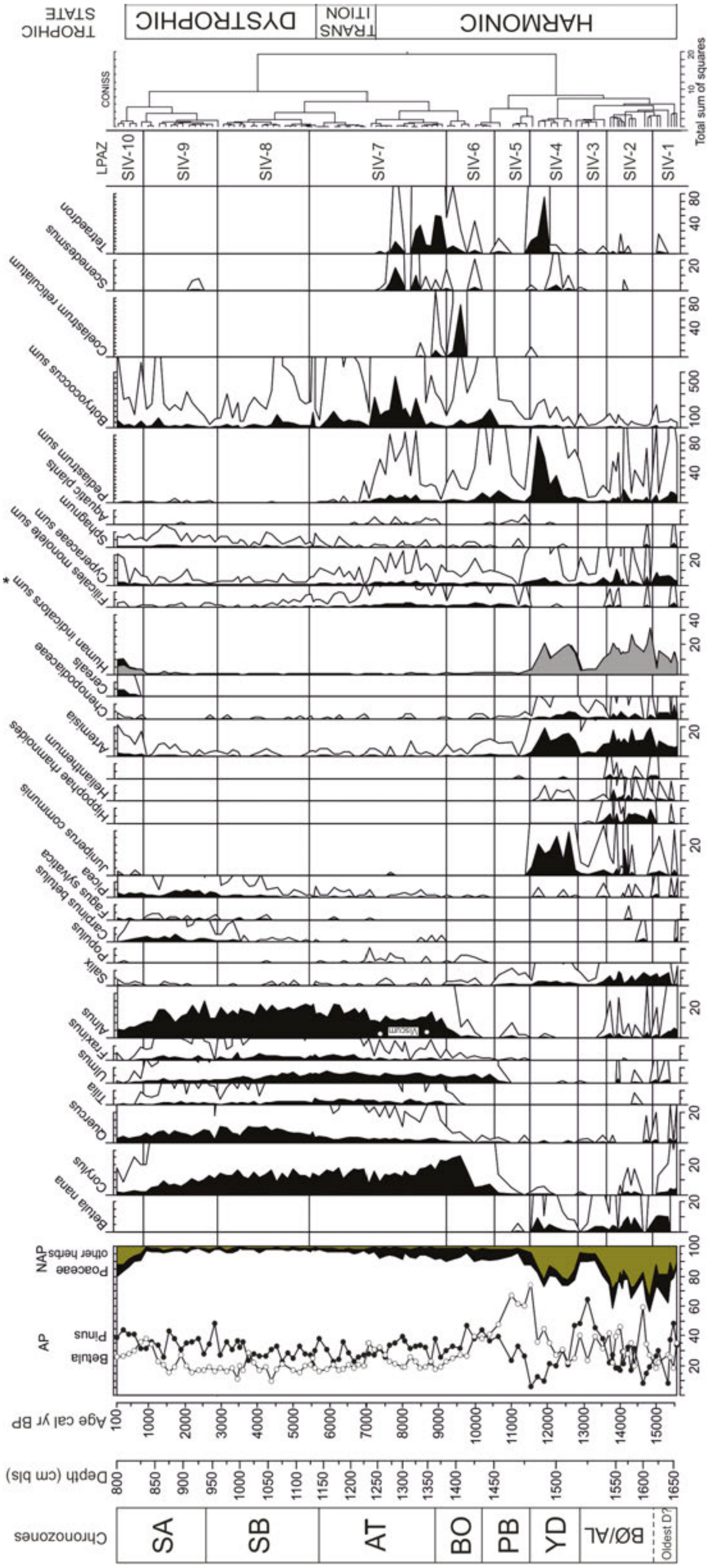


Figure 3. Percentage pollen diagram with selected taxa. Asterisk indicates species present in the environment of Lake Suchar IV before human activity due to climatic condition. *Diagrama del porcentaje de polen con taxones seleccionados. Los asteriscos indican la presencia de especies en el medio del lago Suchar IV antes de la actividad humana debido a condiciones climáticas.*

Pollen analysis

Palynological analyses were conducted on 104 sediment subsamples, accounting for a resolution of 5–10 cm. Palynological samples (1 cm^3 of sediment per sample) were prepared and analyzed using standard methods according to Berglund & Ralska-Jasiewiczowa (1986). Treated samples were stained with safranin and immersed in glycerine, and sporomorphs were counted under a Zeiss microscope at magnifications of 400x and 1000x (immersion oil). At least 500 pollen grains were counted from each sample, with total pollen counts being lower only in bottom samples because of very low pollen concentrations in the deepest part of the core. Identification of pollen grains and spores was based on Faegri & Iversen (1989), Moore *et al.* (1991), Reille (1992), and Beug (2004). Regional pollen assemblages reported as markers of chronostratigraphic units for the studied time period were used to refine the chronology (Fig. 2). The pollen record of LPAZ was divided into zones using CONISS cluster analysis (Grimm, 1987), aiming to facilitate the report of results. During the analysis, non-pollen palynomorphs (green algae) were identified too. The pollen percentage diagram (Fig. 3) was constructed using Tilia and TiliaGraph (Grimm, 1991/2011).

Subfossil Cladocera analysis

Subfossil Cladocera analysis was conducted on 169 sediment subsamples (1 cm^3) that were processed according to Frey (1986). Samples were heated and stirred in a 10 % solution of KOH for 20 min and washed through a 38 µm mesh. The final residue was dissolved in 10 ml distilled water. Aliquots of 0.1 ml of the final solution were mounted on microscope slides that were in turn use to identify cladoceran's remains at magnifications of 100x, 200x, and 400x, using an OLYMPUS BX40 transmitted-light microscope. Two to four slides (minimum of 200 remains) were counted from each sample. All cladoceran remains were counted (head shields, shells, ephippia, postabdomens), and complete individuals were put together from different parts of the body. The identification of Cladocera was based on Szeroczyńska & Sarmaja-Korjonen (2007) and Flössner (2000). The results were plotted as a relative abundance diagram (Fig. 4), using the C2 software (Juggins, 2007). The total number of Cladocera indicated in the diagram (Fig. 4) were standardized to concentration (Cladocera individuals per gram of dry sediment).

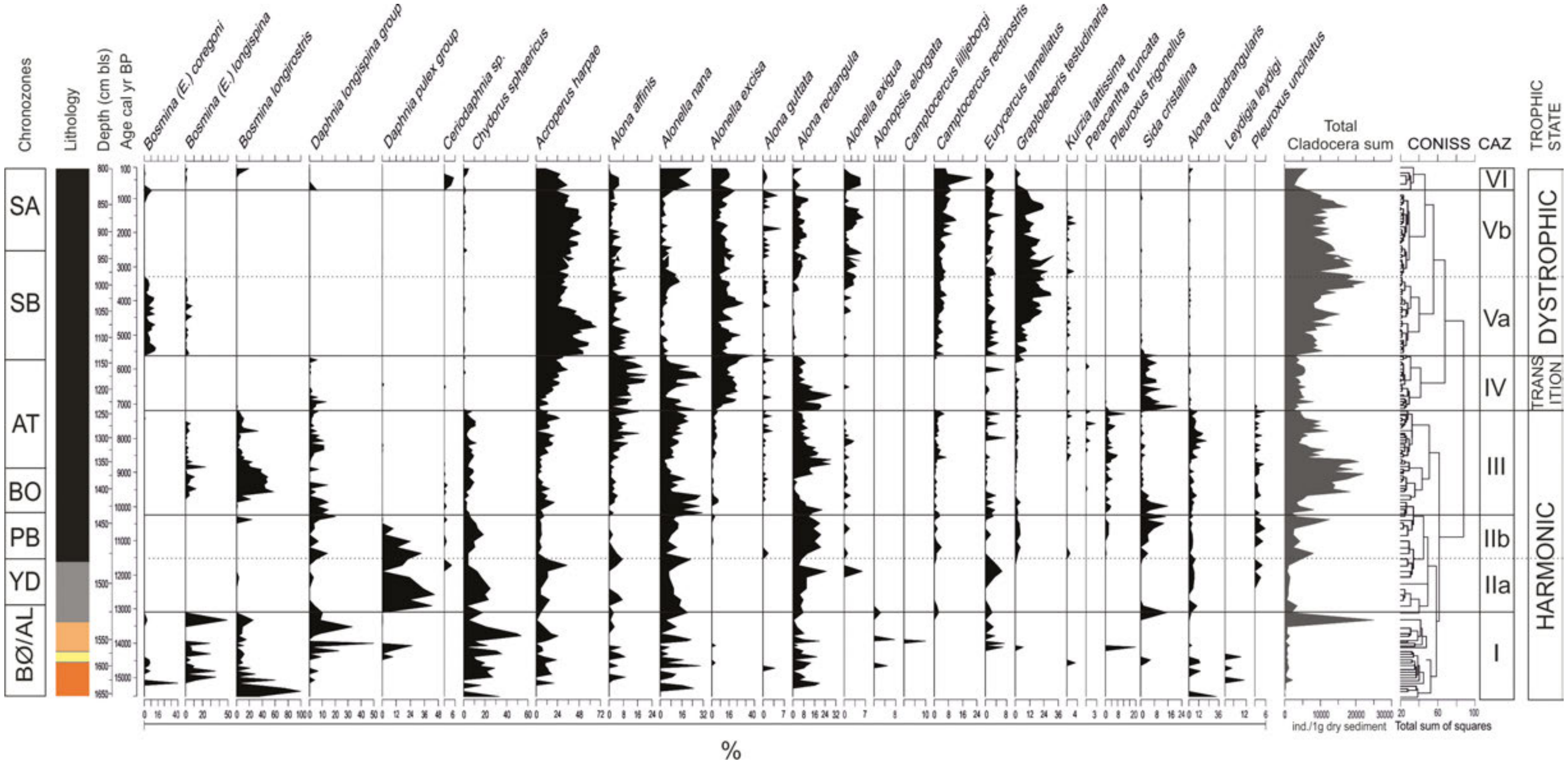
Diatom analysis

Diatom analysis was conducted on 85 sediment subsamples (1 cm^3) according to Battarbee (1986). A 10 % solution of HCl was used to remove calcium carbonate and distilled water was used to wash the samples several times. Afterwards, samples were boiled in 30 % H_2O_2 to oxidize organic matter. Permanent microscope slides were mounted in Naphrax®. The identification of diatoms was performed using a Nikon Eclipse E-200 light microscope at a magnification of ×1000. At least 500 frustules of diatoms were counted on each slide to estimate the relative abundance of each taxon, which were identified according to Krammer & Lange-Bertalot (1986, 1988, 1991a,b), Denys (1991), van Dam *et al.* (1994), Lange-Bertalot & Metzeltin (1996), Lange-Bertalot (1999), Lange-Bertalot & Genkal (1999), Krammer (2002), and Hoffmann *et al.*, (2011). AlgaeBase (Guiry & Guiry 2016) dataset was used for homogenizing taxonomy to the more recent diatom nomenclature. The classification of diatoms into environmental affinities (trophic state, pH), was based on the OMNIDIA database (Lecointe *et al.*, 1993). Also, diatom taxa were grouped according to their lifeform following Round classification (Denys, 1991). The diatom percentages diagram (Fig. 5) was constructed using Tilia and TiliaGraph (Grimm, 1991/2011).

Macrofossils analysis

For macrofossil analysis, 156 samples were washed and sieved through a 125-µm-size mesh and analyzed according to Birks (2007). All macrofossil counts were standardized to concentration (number of individuals per 50 cm^3). Identification of subfossil remains was based on the available literature (Birks, 2007; Mauquoy & van

Figure 4. Relative abundance (%) diagram of subfossil cladoceran taxa identified in the sediments core from Lake Suchar IV, including the CONISS dendrogram on which zonation was based. *Diagrama de la abundancia relativa (%) de los taxones de cladóceros subfósiles identificados en los sedimentos del lago Suchar IV, incluyendo el dendrograma CONISS en el que se basa la zonación.*



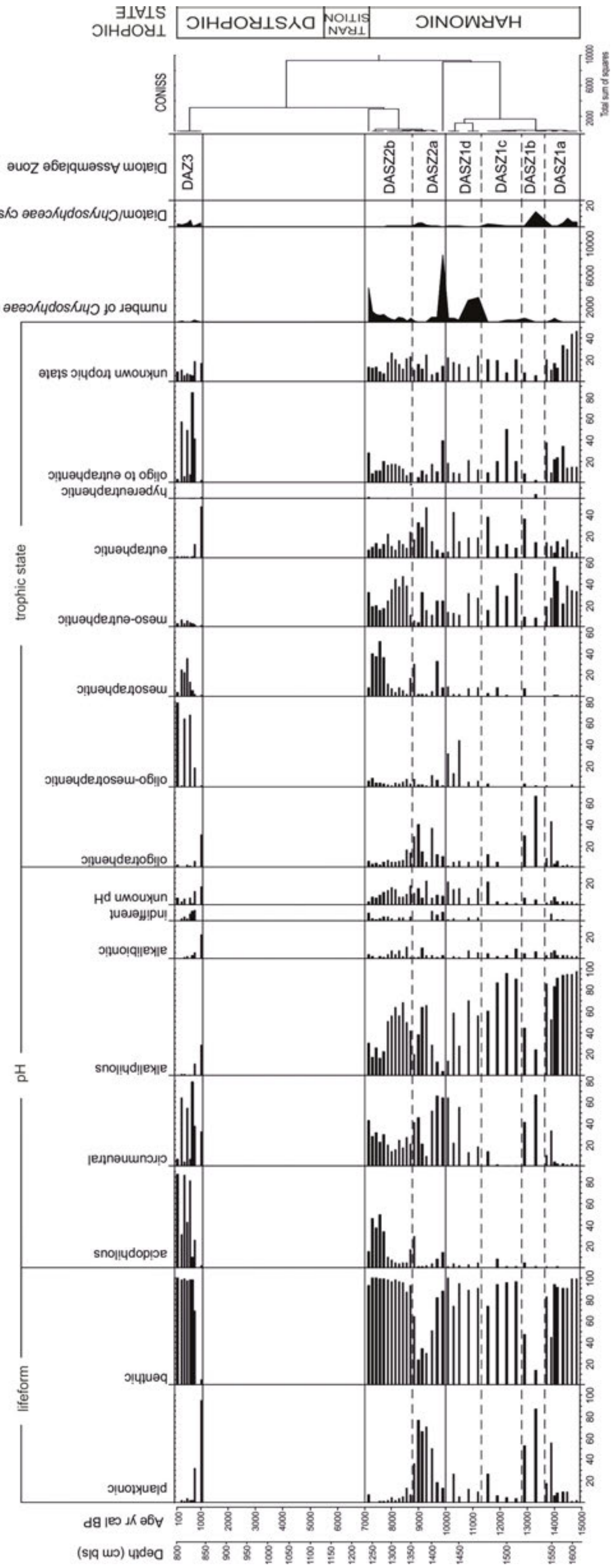


Figure 5. Relative abundance (%) diagram for the diatoms: lifeforms, pH groups, trophic state and Chrysophyceae cysts in the sediments core from Lake Suchar IV including the CONISS dendrogram on which zonation was based. *Diagrama de la abundancia relativa (%) para las diatomeas: formas de vida, grupos según pH, estado trófico y quistes de Chrysophyceae en los sedimentos del core del lago Suchar IV, incluyendo el dendrograma CONISS en el que se basa la zonación.*

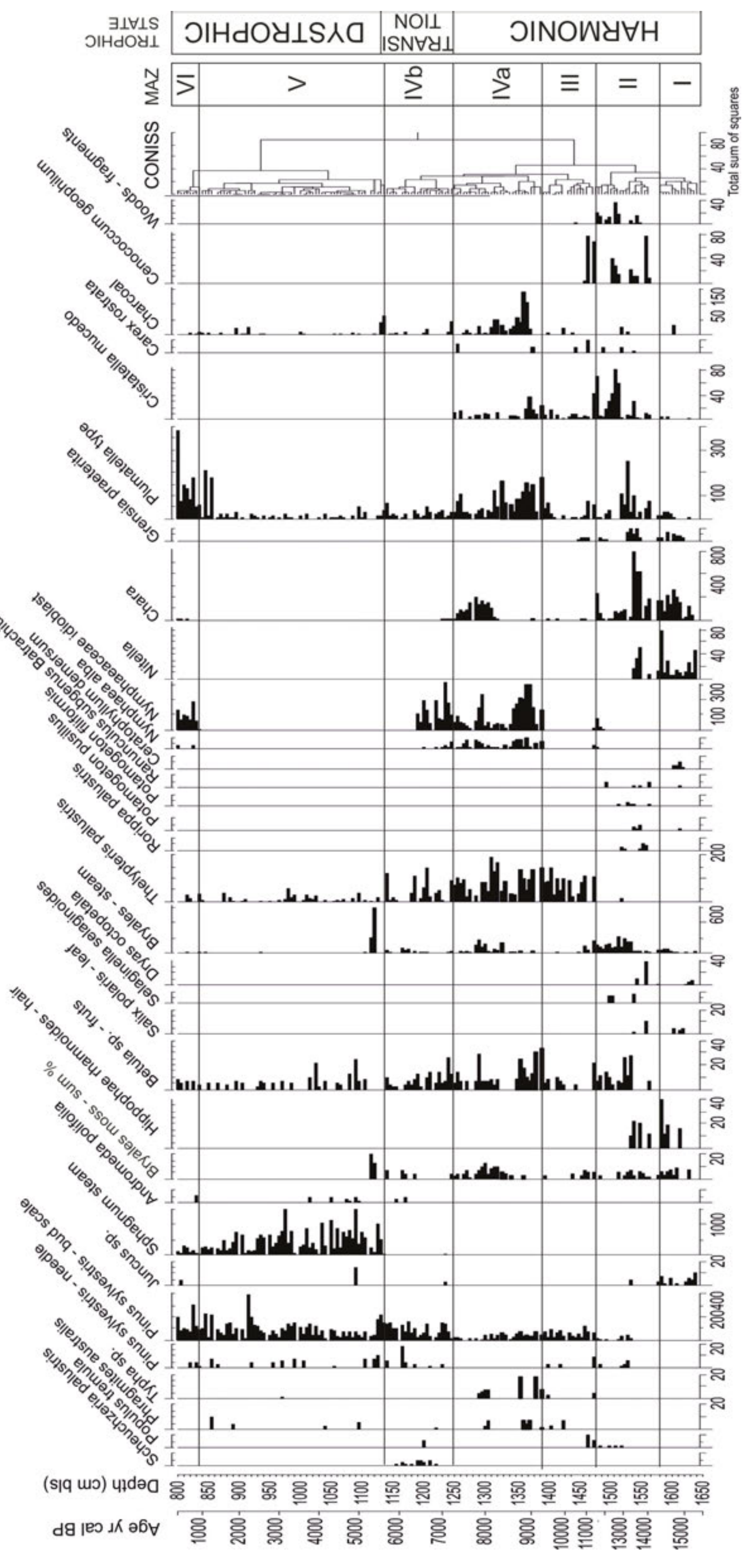


Figure 6. Absolute abundance diagram for macrofossils in the sediments core from Lake Suchar IV including the CONISS dendrogram on which zonation was based. *Diagrama de abundancias absolutas para macrofósiles en los sedimentos del core del lago Suchar IV, incluyendo el dendrograma CONISS en el que se basa la zonación.*

Geel 2007; Velichkevich & Zastawniak 2006; Velichkevich & Zastawniak 2008). The results were plotted using the C2 software (Juggins, 2007) (Fig. 6).

Geochemical analysis

Total carbon (TC), total nitrogen (TN) and total sulfur (TS) were determined in 155 sediment subsamples by Elemental Analysis (EA) (Elementar VarioMax CNS). Total organic carbon (TOC) was determined by analyzing TC in the samples treated with 1 M HCl to remove carbonates. The total inorganic carbon (TIC) was calculated as TIC [wt. %] = TC-TOC. The TOC/N ratio was calculated on a molar basis. All samples were analyzed in duplicate. The quality control was performed using certified reference materials for peaty, chalky and sandy soils, provided by Elementar. The results were plotted (Fig. 7) using the C2 software (Juggins, 2007).

Statistical analysis

A Principal Component Analysis (PCA) was performed based on the correlation matrix of the dataset biological components (algae, cladocerans, and pollen), aiming at generalizing the ecological and environmental information contained in the sedimentary sequence of Lake Suchar IV (Legendre & Legendre, 2012). Before the analysis, the dataset was logarithmically transformed to meet the normality assumption of the method (Borcard *et al.*, 2011). Taxon scores in the correlation biplot were used to interpret the potential relationship among the biological attributes of the dataset. The distance biplot was used to interpret the ecological and environmental meaning of the

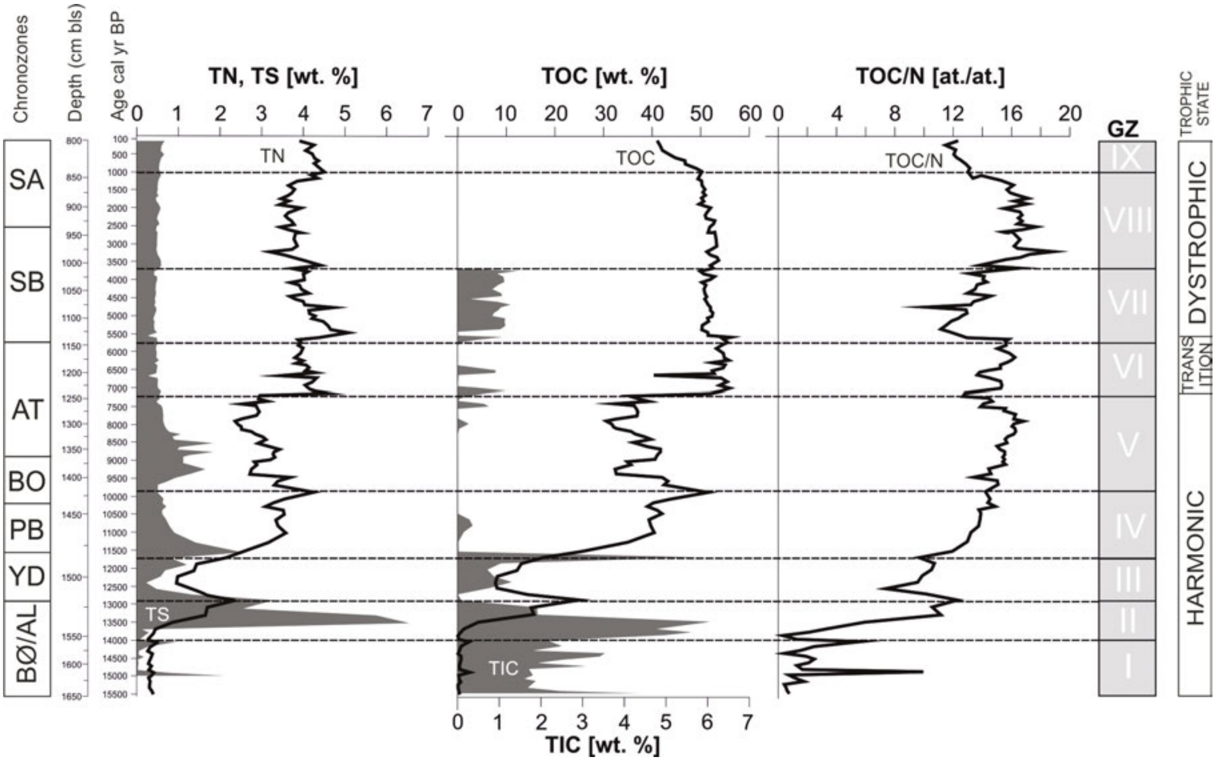


Figure 7. Chemical composition of the sediments of Lake Suchar IV: TN- total nitrogen, TS – total sulphur, TOC- total organic carbon and TIC –total inorganic carbon. *Composición química de los sedimentos del lago Suchar IV: TN - nitrógeno total, TS - sulfuro total, TOC carbono orgánico total y TIC – carbono inorgánico total.*

principal components, whose significance was tested using a broken-stick model.

RESULTS

Sediment lithology

The sediments of Lake Suchar IV were mainly composed of black highly organic sediment probably dy/sapropel (Hansen, 1959). The sediment floor, 1655 cm below lake surface (bls), was composed of sand, with the overlaying layer (1655–1587 cm bls) consisting of fine sand with layers of silt and calcareous gyttja. From 1587 to 1570 cm bls, the sediment was dominated by grey silt gyttja with fine sand. From 1570 to 1530 cm bls, the sediment was composed of beige-grey detritus-calcareous gyttja. From 1530 to 1480 cm bls, the sediment was dark brown detritus gyttja and from 1480 to 800 cm bls, black detritus, unconsolidated "jelly" deposits (dy/sapropel) was identified.

Chronology

Both age-depth models constructed for the sediments of Lake Suchar IV (Table 1) yielded a basal age of ~15 000 cal yr BP. Nevertheless, the models showed inconsistencies in the age of the sediments for the early and late Holocene. Between ~11 000 and 8000 cal yr BP and from 4000 cal yr BP to present, the model based on ^{14}C dates produced ages that were between 100 and 1000 years younger than the model that incorporated chrono-stratigraphic markers. We decided to use the model based on the combination of ^{14}C dates and stratigraphic markers given that the latter have been consistently identified though changes in the region and therefore represent a more parsimonious temporal contextualization for our results.

Pollen

A total of 91 taxa of pollen and spores were identified through the sedimentary sequence of Lake Suchar IV. Based on the results of the palynological analysis, the sediments from Lake Suchar IV have continuously accumulated since the Late Glacial to present. Pollen assemblages suggest that the sediment started accumulating during the Oldest Dryas. Ten local pollen assemblage zones (LPAZ SIV) were distinguished in the percentage pollen diagram, reflecting the stages of vegetation history (Fig. 3). Changes in the occurrence and abundance of green algae and aquatic plants corresponded with the distinguished pollen zones.

LPAZ SIV-1 NAP-*Betula* (The oldest samples before 15 000 cal yr B; 1650÷1615 cm)

High percentage of NAP (above 20 % with max 37 %); *Betula* between 18 and 37 %, *Betula nana* type 10 %. The upper limit marked by the beginning of the continuous curve of *Hippophae rhamnoides*. The presence of *Pediastrum and Botryococcus* colonies and single coenobia of *Tetratedron*.

LPAZ SIV-2 NAP-*Betula-Hippophae* (ca. 15 000–13 500 cal yr BP; 1615 ÷1535 cm)

Betula between 30 and 60 %. The continuous curve of *Hippophae rhamnoides* (max 9 %). High percentage of NAP (21–44 %) with *Artemisia* dominance (8–19 %). Significant contribution of *Helianthemum* pollen grains (max 7 %). The upper limit marked by a decline in NAP and *Hippophae*. The presence of green algae without changes. Only single coenobia of *Scenedesmus* present.

LPAZ SIV-3 *Pinus* (ca. 13 500–12 900 cal yr BP; 1535÷1520 cm)

Rapid growth of *Pinus* till max 67 %. Decline of NAP (< 10 %). Decline of *Salix* (3–1 %). The *Helianthemum* curve disappeared. The upper limit marked by an increase of *Juniperus* and NAP curves. Very low percentage of all chlorophytes.

LPAZ SIV-4 NAP-*Juniperus* (ca. 12 900–11 500 cal yr BP; 1520÷ 1480 cm)

High percentage of *Juniperus* (max 26 %). Increase in NAP (> 20 %) and *Salix* (up to 3 %). *Pinus* declined from 47 to 20 %. Return of the *Helianthemum* curve. The upper limit marked by a rapid decline of *Juniperus* and *Betula nana* t. PAZ with the highest percentage of *Pediastrum* (max 89 %) and *Tetraedron* (75 %). The curve of *Scenedesmus* present.

LPAZ SIV-5 *Betula* (ca. 11 500–10 600 cal yr BP; 1480÷1455 cm)

Increase of *Betula* up to 60 % (max 75 %). Disappearance of *Juniperus* and *Betula nana* t. curves. Decline of NAP below 10 %. The beginning of *Corylus* and *Ulmus* continuous curves. The upper limit marked by an increase in *Corylus* above 5 %. Decline of all green algae.

LPAZ SIV-6 *Corylus* (ca. 10 600–9200 cal yr BP; 1455÷1385 cm)

Pinus and *Betula* below 50 %. Rapid growth of *Corylus* (max 26 %). NAP below 3 %. Poaceae about 7 %. The upper limit marked by the beginning of the continuous curves of *Quercus*, *Tilia*, *Fraxinus* and *Alnus*. Increase of *Pediastrum* and *Botryococcus*. Varying contribution of *Tetraedron* and *Scenedesmus*. High percentage of *Coelastrum reticulatum* in the upper part of LPAZ.

LPAZ SIV-7 *Ulmus* (ca. 9200–5450 cal yr BP 1385÷1125 cm)

AP above 90 %. Pollen grains of the main component of mixed deciduous forest dominate (*Corylus*, *Quercus*, *Tilia*, *Fraxinus*, *Ulmus*, *Alnus*). *Ulmus* between 4 and 8 %. The beginning of the *Picea* curve. The upper limit marked by a decline of *Tilia* and *Ulmus* and increase of *Quercus*. LPAZ with the highest percentage of *Botryococcus*. *Coelastrum reticulatum* disappeared in the bottom part of PAZ. *Pediastrum*, *Scenedesmus* and *Tetraedron* colonies were present till the middle part of the zone and then disappeared

LPAZ SIV-8 *Corylus-Quercus* (5450–2900 cal yr BP; 1125÷960 cm)

Significant growth of *Quercus* (from 5 % to max 11 %.). Decline of *Tilia* and *Ulmus*. The beginning of the continuous curve of *Carpinus*. The upper limit marked by an increase of *Picea* and *Carpinus* curves. Only *Botryococcus* occurred in the water.

LPAZ SIV-9 *Picea-Carpinus* (2900–800 cal yr BP; 960÷830cm)

The growth of *Picea* (up to 6 %) and *Carpinus* (up to 5 %). Small increase in the percentage of *Pinus*. Gradual decline of *Corylus*. The upper limit marked by an increase in NAP. Small contribution of *Botryococcus* and single appearance of *Pediastrum* and *Scenedesmus*

LPAZ SIV-10 *Pinus*-NAP (ca. 800–100 cal yr BP; 830÷800 cm)

Significant growth of NAP (9–20 %). Human indicators present, e.g. pollen grains of cereals. Decline of deciduous tree curves. Low percentage of *Botryococcus* and single coenobia of *Pediastrum*.

Subfossil Cladocera

A total of 36 Cladocera species belonging to five families were identified in the sedimentary sequence of Lake Suchar IV. Planktonic species were represented by the families of Bosminidae and Daphniidae, and littoral species by Chydoridae, Holopedidae and Sididae. Littoral species represented the major (~60 %) component of the Cladocera assemblages, especially through the last 7000 years (Fig. 4). Cladocera assemblages composition and structure allowed the identification of six Cladocera assemblage zones (CAZ) that summarize the main stages of Cladocera development in Lake Suchar IV (Fig. 4). Cladocera concentrations during the Late Glacial was very low (except for the Allerød), increasing substantially during the Holocene.

CAZ I (The oldest samples from origin till 13 100 cal yr BP; 1655÷1525 cm)

Twenty-one species were identified in the sediments. The total number of Cladocera individuals per 1 g of dry sediment was ca. 1700. Planktonic species from Bosminidae and Daphniidae dominated. *Chydorus sphaericus*, *Acroperus harpae* and *Alonella nana* dominated in the littoral.

CAZ II (ca. 13 100–10 300 cal yr BP; 1525÷1440 cm)

Twenty Cladocera species were identified. The number of Cladocera individuals increased to 3400. Specimens from the Daphniidae family dominated (on average 22 %) among planktonic species: the *Daphnia longispina* group and the *Daphnia pulex* group. Bosminidae almost completely disappeared. *Alona rectangula* (on average 12 %), *Chydorus sphaericus* (on average 11 %) and *Alonella nana* (on average 11 %) were most abundant in the littoral.

CAZ III (ca. 10 300–7200 cal yr BP; 1440÷1245 cm)

The number of Cladocera individuals (average 10 000; max 22 000) and the number of species (23) significantly increased. Planktonic species accounted for 25 % on average. *Bosmina longirostris* (max 58 %) was the most abundant one. Littoral species were dominated by *Alonella nana* and *Alona rectangula*.

CAZ IV (ca. 7200–5600 cal yr BP; 1245÷1135 cm)

The number of Cladocera individuals (4300) and the number of species (16) decreased. The percentage of littoral species (~98 %) significantly increased; *Acroperus harpae*, *Alona affinis*, *Alona rectangula*, *Alonella nana* and *Alonella excisa* dominated. *Chydorus sphaericus* completely disappeared. Among planktonic species, only the *Daphnia longispina* group was present but with very low percentage – on average 2 %.

CAZ V (ca. 5600–750 cal yr BP; 1135÷830 cm)

The number of Cladocera individuals significantly increased (average 11 800). Littoral taxa dominated, especially species associated with macrophytes: *Acroperus harpae* (41 %), *Graptoleberis testudinaria* (15 %) and acidophilous *Alonella excisa* (14 %). Planktonic species occurred again (average 5 %): *Bosmina (E.) coregoni* and *Bosmina (E.) longispina*.

CAZ VI (ca. 750–100 cal yr BP; 830÷800 cm)

The number of Cladocera individuals decreased (average 4800). The importance of planktonic species slightly increased: Bosminidae and Daphniidae (mean 10 %). *Acroperus harpae*, *Alonella nana* dominated in the littoral.

Diatoms

A total of 193 diatom taxa (species and varieties) were identified. The sequence was divided into 3 diatom assemblage zones (DAZ), two of which were subsequently divided into subzones (DASZ) (Fig. 5). The diatom zones were defined using CONISS – the cluster analysis software (Grimm 1991/2011).

DASZ 1a (The oldest samples from origin till 13 700 cal yr BP; 1655÷1540cm)

Absolute dominance of benthic diatoms (*Fragilaria lapponica*, *Pseudostaurosira brevistriata*, *Staurosira construens*, *Staurosirella pinnata*). Dominance of alkaliphilous species; high proportion of oligotraphentic and eutraphentic species. Increase in contribution of diatoms/Chrysophyceae cysts (D/Ch) indicator from 0.4 to 7.8.

DASZ 1b (ca. 13 700–12 900 cal yr B; 1540÷1520 cm)

Abundance of planktonic species increased and then declined (*Lindavia comensis*, *Cyclotella ocellata* and *Puncticulata radiosa*). Dominance of circumneutral species; high proportion of oligotraphentic taxa. The content of D/Ch indicator increased up to 14.

DASZ 1c (ca. 12 900–11 500 cal yr BP; 1520÷1480 cm)

Decline of planktonic taxa; increase in benthic species, mainly *Fragilaria lapponica*, *Pseudostaurosira brevistriata*, *Staurosira construens*, *Staurosirella pinnata*. Dominance of pH alkaliphilous. Dominance of meso-eutraphentic taxa; high values of eutraphentic taxa. Decrease in D/Ch indicator (average 1.6).

DASZ 1d (ca. 11 500–10 000 cal yr BP; 1480÷1430 cm)

Dominance of benthic diatoms, mainly *Staurosira construens*, *Navicula cryptocephala*, *Navicula radiosa*. Dominance of pH circumneutral, followed by alkaliphilous taxa. High values of meso-eutraphentic, eutraphentic and mesotraphentic taxa. First and large increase of Chrysophyceae cysts. D/Ch indicator values decrease to 0.3.

DASZ 2a (ca. 10 000–8800 cal yr BP; 1430÷1360 cm)

Decline of benthic taxa; increase in planktonic species (*Lindavia comensis*, *Cyclotella ocellata*, *C. meneghiniana* and *Puncticulata radiosa*). Dominance of pH alkaliphilous; low proportion of circumneutral diatoms. High contribution of taxa with a wide range of tolerance (oligo- to eutraphentic). The largest increase in the number of Chrysophyceae cysts. The D/Ch indicator was rather low.

DASZ 2b (ca. 8800–7100 cal yr BP; 1360÷1240 cm)

Decline of planktonic taxa; increase in the number of benthic species, mainly *Staurosira construens*, *Navicula radiosa*, *Pinnularia gibba*, *Stauroneis phoenicenteron*. Dominance of circumneutral taxa and alkaliphilous; increase in acidophilous taxa. High values of mesotraphentic, meso-eutraphentic and eutraphentic taxa.

Lack of diatoms (ca. 7100–1100 cal yr BP; 1240÷845 cm)

DAZ 3 (ca. 1100–100 cal yr BP; 840÷800 cm)

Dominance of planktonic and then benthic species (*Pinnularia subcapitata* var *elongata*, *Pinnularia gibba*, *Tabellaria flocculosa*, *Stau-*

roneis phoenicenteron). Increase in acidophilous taxa; large number of circumneutral species. Dominance of oligo-mesotraphentic taxa; large numbers of mesotraphentic, meso-eutraphentic and eutraphentic taxa. High contribution of diatoms/Chrysopohyceae cysts: 0.6–6.4.

Macrofossil

In the studied sediment, were identified macrofossil remains of 48 taxa and were dominated mostly by water plant species (submerged and floating plants) and wetlands species connected with overgrowing mats. Results allowed to distinguish six macrofossil assemblage zones (MAZ) which are shown by an absolute frequency composition diagram (Fig. 6).

MAZ I (the oldest samples ~15 000- 14 400 cal yr BP; 1640÷1585 cm)

Algae from the family Characeae (*Chara sp.* and *Nitella sp.*) dominated in the open water zone. *Ceratophyllum demersum*, *Potamogeton pusillus*, *Ranunculus sect. Batrachium* occurred in the littoral zone. Reed beds occurred in the shore zone and they were dominated by *Juncus sp.* and *Bryales* sp. The vegetation development was accompanied by increased abundance of the bryozoans: *Plumatella type* and *Cristetella mucedo*. *Salix polris*, *Hippophae rhamnoides*, *Betula nana*, *Dryas octopetala* grew in the nearest vicinity of the lake.

MAZ II (ca. 14 400–11 500 cal yr BP; 1585÷1480 cm

Nitella sp. and *Chara* sp. co-occurred in the form of underwater meadows. *Nitella* sp. disappeared in the middle of this period, along with species from the *Potamogeton* genus. The relative abundance of the *Plumatella* type and *Cristetella mucedo* increased. The arrival of pine and birch was followed by the withdrawal of *Juncus sp.*, *Salix polris*, *Selaginella selaginoides*, *Hippophae rhamnoides*, *Betula nana*, *Dryas octopetala*. The amount of charcoal and the abundance of *Cenococcum geophilum* increased. *Populus tremula*, *Rorippa palustris* occurred and Nymphaeaceae in the planktonic zone.

MAZ III (ca. 11 500-9300 cal yr BP; 1480÷1390 cm)

The decline or lack of some aquatic species (e.g.*Chara* sp) was accompanied by a decline of *Plumatella* sp. and *Cristatella mucedo*. The littoral zone was dominated by *Thelypteris* sp. The percentage of birch decreased, *Typha* sp. and *Phragmites australis* occurred.

MAZ IVa (ca. 9300-7100 cal yr BP; 1390÷1240 cm)

Dominance of Nymphaeaceae (*Nymphaea alba*). *Chara* sp. occurred again in the second part of this phase and was accompanied by *Plumatella* sp. *Cristetella mucedo* disappeared at the end of this zone. The amount of wood charcoal increased.

MAZ IVb (ca. 7100-5700 cal yr BP; 1240÷1135cm)

The increased content of Nymphaeaceae was accompanied by disappearance of *Chara* sp. and *Cristetella mucedo*. The percentage of pine increased. The amount of charcoal and the content of *Bryales* sp. macrofossils decreased.

MAZ V (ca. 5700-1000 cal yr BP; 1135÷840 cm)

Floating mats of *Sphagnum* developed in the littoral zone, which was accompanied by the disappearance of *Bryales* sp. and Nymphaeaceae.

MAZ VI (ca. 1000-100 cal yr BP; 840÷800 cm)

The decline of *Sphagnum* and the return of Nymphaeaceae in the near-shore zone. The increased abundance of *Plumatella* sp.

Geochemistry

Nine geochemical zones (GZ) were identified on the basis of changes in TS, TN, TOC, TIC and TOC/N. The highest content of nitrogen was identified in the second half of the Holocene – from the transition state (ca. 7200 cal yr BP), accompanied by the maximum content of Total Organic Carbon (TOC). The highest content of sulfur was recorded during the Allerød (GZ II), along with the maximum content of Total Inorganic Carbon (TIC). Since the transition time, the content of sulfur remained below 1 %. A high content of TIC (up to 10 %) was also found in the early Subboreal period (GZ VII) (Fig. 7). Based on the observed changes in TOC, TIC and TN and TS, nine Geochemical zones (GZ) were distinguished:

GZ I (The oldest samples before 15 000 – 14

000 cal yr BP; 1655÷1565 cm)

Sediments are predominantly mineral with very low TOC and considerable carbonate content (2–3 % TIC). Low TOC/N indicates autochthonous organic matter.

GZ II (ca. 14 000–12 900 cal yr BP 1565÷1520 cm)

Sediments are distinguished by the high TIC (up to ca. 6 %) and TS (up to 6.5 %). TOC and TOC/N are increasing toward the top of the zone. Increasing TOC/N (up to 12) indicates the increasing contribution from terrestrial (soil-derived, macrophytes) organic matter.

GZ III (ca. 12 900–11 700 cal yr BP; 1520÷1485 cm)

Sediments are characterized by low TOC and TIC, but increasing toward the upper layer. TS content is low < 1 %. The reduced TOC/N ratio indicates the predominance of phytoplankton-derived organic matter.

GZ IV (ca. 11 700–9900 cal yr BP; 1485÷1420 cm)

Sediments are characterized by steeply increasing TOC and decreasing TS. TIC is depleted, except for the bottommost section of the zone. TOC/N gradually increases to ca. 15.

GZ V (ca. 9900–7200 cal yr BP; 1420÷1245 cm)

TOC decreases sharply and TIC is completely absent. TS shows enrichment between 9500–8200 cal yr BP. TOC/N is high and increases throughout the zone, except for the top section.

GZ VI (ca. 7200–5700 cal yr BP; 1245÷1145 cm)

TOC shows maximum values throughout the core and increases sharply at the base of the zone. TOC/N is high and TIC is absent.

GZ VII (ca. 5700–3800 cal yr BP; 1145÷1015 cm)

TOC/N shows a decline from ca. 16 to ca. 8. This coincides with a slight increase in TIC to ca. 1 %. TS is low (< 1 %). TOC is high (ca. 50 %).

GZ VIII (ca. 3800–1000 cal yr BP;1015÷840 cm)

The zone is characterized by high and invariant TOC (ca. 50 %). TIC is absent, TS is low and TOC/N shows the highest values throughout the core (14–20).

GZ IX (ca.1000–100 cal yr BP; 840÷800 cm)

TOC and TOC/N decrease. TIC is absent and TS is low.

Principal components analysis

The dataset used for the ordination contained a total of 154 taxa that yielded a log-transformed variance of 31.41 units. PCA 1 and 2 axes explained 33.3 and 15.5 % of the total variance, respectively, and resulted significant according to the broken-stick model. Extreme scores in the correlation biplot were obtained by algae and cladocerans (Fig. 8), reflecting the higher variability inherently linked with indicators derived from short-lived organisms. PCA 1 axis sample scores were around -1.0 between the bottom of the record and ~11 500 cal yr BP (Fig. 8). From ~10 500 to 5500 cal yr BP, PCA 1 axis sample scores progressively raised to reach scores of ~0.8, which in turn prevailed up to present. PCA 2 axis sample scores progressively fell from ~2.0 at the bottom of the sequence to ~1.5 at ~7500, when they started increasing up to ~0.3 at ~5500 cal yr BP. From ~5500 to present, Axis 2 sample scores remained relatively stable around 0.3.

DISCUSSION

With a basal age that goes back to the Oldest Dryas, the sedimentary sequence of Lake Suchar IV enabled us to reconstruct climatic and ecological changes occurred in the lake and its catchment through the last ~15 000 years. Paleolimnological studies enabled us detailed tracing of transformation processes from the harmonic into disharmonic (dystrophic) state, a change pattern that has been the focus of limnological and paleolimnological studies (e.g. Sayer *et al.*, 2010; Smol, 1992). Although the concept and the state of dystrophy remain ambiguous and controversial (e.g. Alhonen, 1987; Havens, 1991; Marek, 1992; Carpenter & Pace, 1997), most researchers understand dystrophic lakes as low-primary-production oligotrophic water bodies. The high contents of organic carbon and humic acids in these lakes make the water acid and dark brown (Alhonen, 1987; Marek, 1992; Górniak, 2006).

The lithological variability of the sedimentary sequence (gyttja, calcareous gyttja, dy/sapropel)

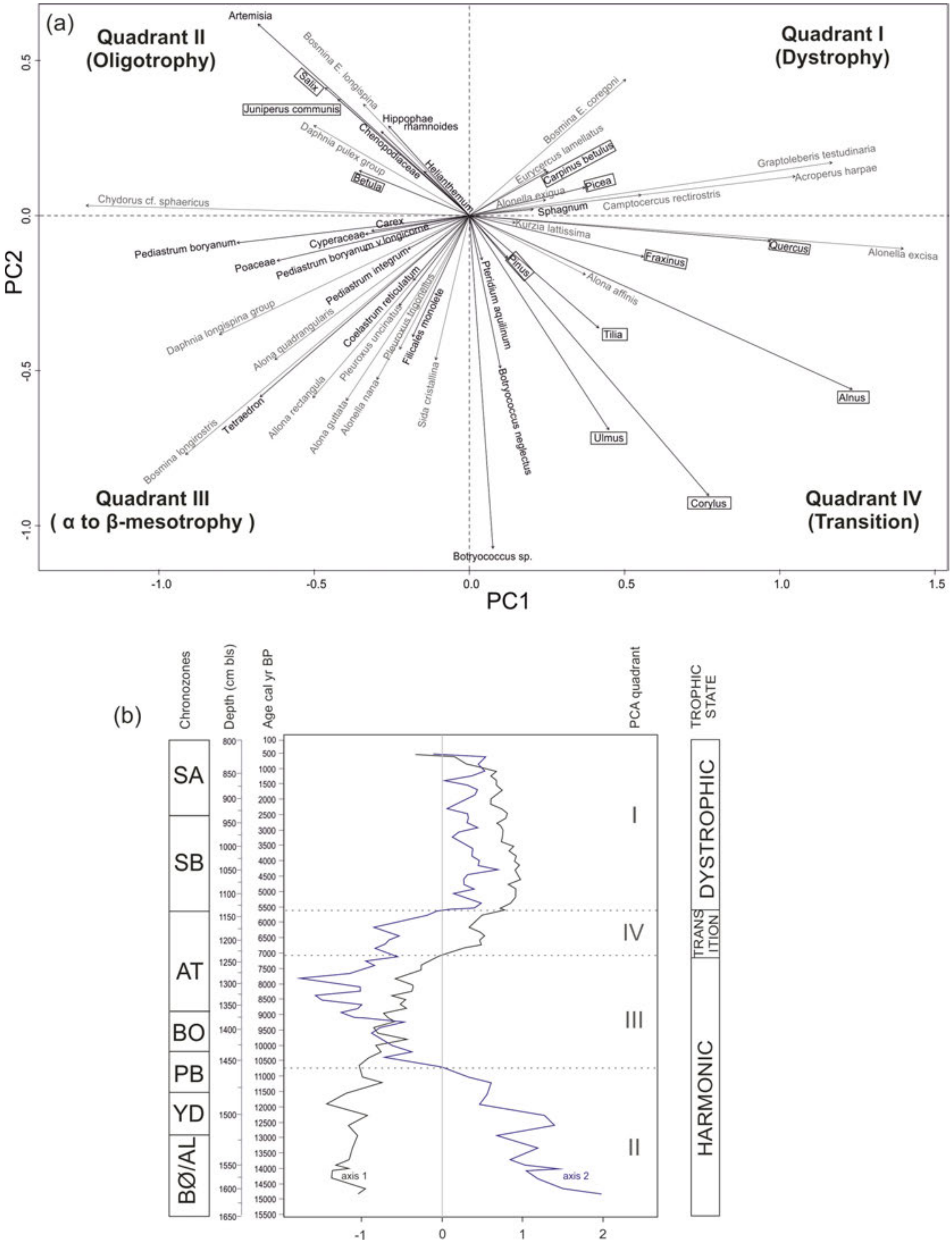


Figure 8. a) Principal component analysis (PCA) biplot of biological components (green algae, cladocerans, pollen) in the sediment core of Lake Suchar IV; b) PCA axis 1 and axis 2 scores for samples following either their algae, cladoceran and pollen assemblages (percentages) in the sediment core of Lake Suchar IV. *a) Análisis de componentes principales, PCA biplot, de los componentes biológicos (algas verdes, cladóceros y polen) en los sedimentos del core del lago Suchar IV; b) valores del eje 1 y eje 2 del PCA para muestras que siguen cualquier asociación (porcentaje) de algas, cladóceros y polen en los sedimentos del core del lago Suchar IV.*

and changes in its chemical composition (TN, TS, TIC, TOC, TC/N), demonstrate that the history of Lake Suchar IV has been intimately linked to regional environmental conditions. The inflow of biogenic and humic substances from the catchment determined both the type of sediments and the trophic conditions of the water body. Substantial changes in the proportions of major plant types and species composition of cladocerans and diatoms suggest a successional pattern that reflect the changes in the lake. Both phyto- and zooplankton responded not only to changes in water temperature and trophic state (oligotrophy → α-mesotrophy →β-mesotrophy →dystrophy) (Fig. 9) associated with the deglaciation, but also to the degree of humification of the water body (pH and color of water). Today, the high content of humic substances in the water of Lake Suchar IV results in water with a dark brown coloration that reduces the penetration of sunlight into deeper water layers (0.65 m Secchi disk). According to the fossil evidence, the lake acquired this attribute at ~5600 cal BP. The changes of Lake Suchar IV could be broadly summarized in three stages marked by significant changes of fauna and flora. From the bottom of the record up to 7200 cal yr BP the evidence reflects a harmonic development of the lake. Significant ecological changes occurred in Lake Suchar IV during the second part of the Atlantic period (7200–5600 cal yr BP) that transformed the lake into the modern disharmonic (dystrophic) state (5600 cal yr BP to present).

PCA sample scores show changes in the lake and the regional system that describe the sequence of dystrophication. Samples from the bottom of the sequence up to 11 500 cal yr BP resulted ordinated obtained negative and positive scores in PCA 1 and 2 axes, respectively (PCA quadrant II, Fig. 8). This quadrant was occupied by vegetation taxa typical of the deglaciation and pioneer cladocerans species typical of oligotrophic lakes. From 11 500 to 7200 cal yr BP, samples were ordinated in quadrant III of the PCA (negative scores for both axes, Fig. 8), which was char-

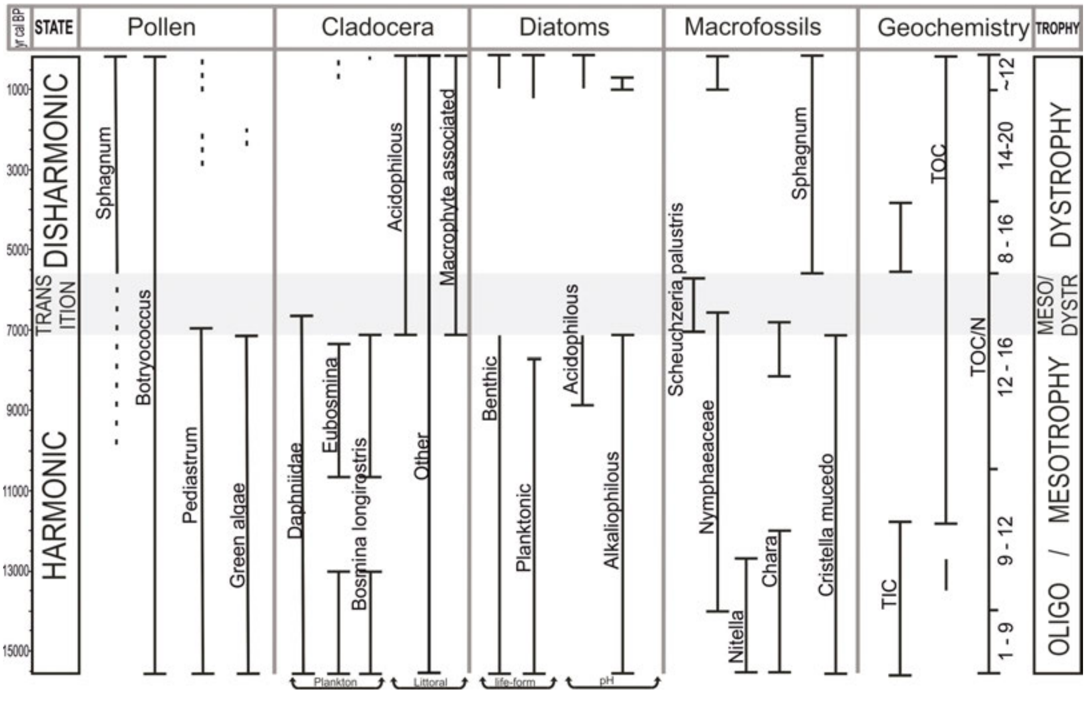


Figure 9. A Comparison of selected sequences of: pollen, Cladocera, diatoms, macrofossils and geochemistry, in the sediment core from Lake Suchar IV. *Una comparación de secuencias seleccionadas de: polen, Cladócera, diatomeas, macrofósiles y geoquímica, en los sedimentos del core del lago Sucher IV.*

acterized by algae and cladocerans typical of α to β-mesotrophic environments. This phase coincides with the development of a more thermophilous system at local and regional scales. From 7200 to 5600 cal yr BP, sample scores in both axes increased rapidly, although remained mostly within quadrant IV of the ordination. The rapid pace at which these changes took place suggest a transitional state of the lake that led to positive scores in both axes (PCA quadrant I, Fig. 8) from ~5600 cal yr BP and present. This quadrant was dominated by algae and cladoceran species typical of dystrophic environments in regional forests appeared Picea, and a water body with abundant floating mats of *Sphagnum*. Overall, the PCA suggests a sequence from oligotrophic to α- to β-mesotrophic to dystrophic. The initial changes from oligotrophic to mesotrophic is considered a harmonic sequence, whereas the dystrophic zone is considered disharmonic. Although there was an evident change in the local and regional conditions at the Pleistocene-Holocene transition, our interest is focused on the dystrophication process, and therefore our discussion is structured in three phases articulated around the transition that took place between 7200 and 5600 yr cal BP.

Harmonic stage (*from the initial time to 7200 cal yr BP*)

From the Oldest Dryas to ~7200 cal yr BP (i.e. till the mid-Atlantic period), Lake Suchar IV was apparently characterized by a harmonic development (Fig. 9). Sediments deposited during the Late Glacial significantly varied in terms of lithology (sand, calcareous gyttja) and the content of TIC and TOC, indicating relatively frequent fluctuations related to climate change (Ralska-Jasiewiczowa *et al.*, 1998). High concentration of TIC during the Late Glacial suggest that the sediments were mostly allochthonous. In slightly warmer periods (the Bølling and the Allerød), the content of TOC was higher, while during the colder periods (the Oldest, Old and Younger Dryas) –TIC and TS contents increased. The species composition of flora and fauna deposited during colder periods indicates that the lake was shallow at that time and its waters were oligotrophic. *Chrysophyceae* cysts, charophytes and diatoms occurred at that time, especially alkaliphilous species (*Pseudostaurosira brevistriata* and *Stauroneis construens*). The cladocerans were represented both by littoral and planktonic species. In warmer periods (the Bølling and the Allerød), the content of oligo/mesotrophic taxa (e.g. Daphniidae, *Chydorus sphaericus*, *Pleuroxus*), in the plankton slightly increased. Aquatic vegetation was represented mainly by *Potamogeton* and reed beds in the shoreline zone of the lake. During the Late Glacial, the most favorable temperature and edaphic conditions for the development of plankton prevailed in the lake during the late Allerød when the zooplankton frequency reached the maximum value (Total Cladocera sum; Fig. 4). In the PCA, these conditions are reflected by low and relatively stable scores of Axis 1, and declining and highly variable scores in Axes 2 (Fig. 8). A similar sequence of changes was determined by Drzymulska *et al.* (2014) in Suchar Wielki located in Wigry National Park. In general, the recorded sequence of changes in sediments of Suchar IV in the Late Glacial period is typical of oligotrophic lakes of N Poland, formed after the ice sheet melting (Zawisza & Szeroczyńska, 2007; Filoc *et al.*, 2017). As evidenced by other studies from the late post-glacial areas of N Poland (e.g. Ralska-Jasiewiczowa *et al.*, 1998; Zawisza & Szeroczyńska, 2007), small water bodies formed in depressions after melting of dead-ice blocks were initially oligotrophic and gradually evolved following the climate fluctuations toward mesotrophy representing the harmonic development.

The beginning of the Holocene is reflected in the significant change of arboreal pollen spectra. The open plant communities with the juniper shrubs and light demanding species (e.g. *Helianthemum*, *Artemisia*) began to replace by forest communities. At first with *Betula*, than *Pinus* and new spreading deciduous trees (*Corylus*, *Ulmus*, *Alnus*). In the area around the lake developed the local communities of *Thelypteris palustris* that caused a significant inflow of nitrogen (TN) and organic carbon (TOC) into the lake. This vegetation cover change is clearly reflected in the lowest scores of PCA 2 axis. Such changes probably resulted in the gradual increase in the trophic status of the lake. Diatoms were increasingly represented by meso-eutraphentic and eutraphentic species such as *Staurosira construens*, *Navicula radiosa*, *Pinnularia gibba* and *Stauroneis phoenicenteron*. The harmonic development of the lake continued, with a species composition of cladocerans and green algae similar to that determined in the Late Glacial, although taxon abundances were much higher (Figs. 3 and 4). At the beginning of the Holocene, *Nitella* and *Chara* disappeared from the lake, indicating that waters of the lake changes of water trophy transparency or water depth (Hannon & Gillard, 1997). The harmonic development of the lake, from the oligotrophic state through α-mesotrophy to β-mesotrophy continued until the mid-Holocene Climate Optimum (Atlantic), when abundances of both phyto- and zooplankton specimens reached the highest values. A gradual reduction in the phyto- and zooplankton species in Lake Suchar IV occurred in the second half of the Atlantic period (Figs. 3 and 4). The observed sequence of changes during the Late Glacial period and the first half of the Holocene in Lake Suchar IV is typical of shallow lakes in Poland. During the climate optimum, these lakes were usually meso- and eutrophic, and were often transformed into land or peat bogs (Żurek, 1994; Kowalewski, 2014). Significant changes in the aquatic ecosystem of Lake Suchar IV occurred also at the end of the Atlantic period, when the development of the lake changed from harmonic into disharmonic. The occurrence of acidophilous diatoms, *Sphagnum moss* (Fig. 3) and the gradual increase in the frequency of *Alona guttata* and *Alonella excisa* were indicative of the changing water pH and a harbinger of significant ecological changes. These changes are reflected in the progressive increasing of PCA 1 scores and decreasing PCA 2 scores (Fig. 8).

Transitional stage (*7200–5600 cal yr BP*)

Major changes occurred in the lake during this period (Fig. 9), while the vegetation and climate remained relatively stable (Fig. 3). Species such as *Nymphaea alba*, *Chara*, *Typha*, algae (*Scenedesmus*, *Tetraedron*) and *Pediastrum* completely disappeared from the lake. The species composition of phyto- and zooplankton changed significantly. Frequency of Cladocera specimens was very low and planktonic species were replaced by littoral ones (Fig. 4). The littoral species were dominated by acid-tolerant *Alonella excisa* and species occurring among vegetation (*Alona affinis*, *Sida crystallina*). At that time diatoms totally disappeared. The periphytic fauna (creeping on surface), i.e. *Cristatella mucedo* also completely disappeared during that period. The content of *Bryales* was minor, while the interesting fact is that *Scheuchzeria palustris* occurred only during the transition state. Probably, this species was involved in the creation of mire on the lake shore, which characterize wet conditions (Słowiński *et al.*, 2016). The sediments deposited during the transition state are characterized by an increased content of TN, while TIC and TOC fluctuated significantly. The TOC/N ratio increased to a value of 16. This indicates a change in the trophic conditions of the lake toward dystrophy (Rosen, 2005; Rantala *et al.*, 2015). It is likely that the lake in the transition period (7200–5600 cal yr BP) was relatively shallow, and its waters were characterized by lower pH, reduced transparency and a low content of nutrients. These changes were reflected by increasing trends in the scores of the two first PCA axes (Fig. 8).

Disharmonic (Dystrophy) stage (*5600 cal yr BP to present*)

The end of the Atlantic and the beginning of the Subboreal periods was marked by major climatic changes (cooler weather and increased moisture content) that were clearly reflected in the sediments of Lake Suchar IV. At the beginning of the Subboreal period, the water level of lake Suchar IV likely increased, enabling the re-colonization of the lake by planktonic cladocerans from the family of Bosminidae. The littoral zone of the lake was inhabited by Cladocera species living in associations with aquatic vegetation and tolerant of reduced water pH (mainly Alonidae, *Acroperus harpae*, and *Graptoleberis testudinaria*) (Fig. 4). Zooplankton reached the maximum development at that time, and fluctuations in the TOC/N ratio (8 - 20) indicate that both allochthonous and autochthonous materials were important sources of material to the lake (Figs. 7 and 9). The sediments deposited in the first half of the Subboreal period contained approximately 10 % TIC, indicating a significant inflow of allochthonous material from the catchment (Alhonen, 1987). Climate conditions and the continued development of *Sphagnum* intensified the initiated change in phyto- and zooplankton succession. The conditions in the lake were becoming polyhumic. A similar picture of changes and humic conditions in boreal (Polish and Finnish) lakes was recorded by Drzymulska *et al.* (2015), Luoto *et al.* (2013) and Rantala *et al.* (2015). The intensive development of *Sphagnum* mat on the shore and *Botryococcus* brought about a consistent systematic change in the structure of Lake Suchar IV. The invasion by *Sphagnum* created surface platforms in the form of floating mats which affected the regular development of the lake, especially in the open water zone. From about 3000 to ca 500 cal yr BP, only the littoral zone was inhabited by oligo and/or acid-tolerant species of phytoplankton (pioneer diatoms: *Lindavia comensis*, *Cyclotella planktonica*, *Puncticulata radiosa*) and zooplankton (Alonidae e.g. *Alona guttata*, *Alonella exigua*; *Acroperus harpae*). The development of *Botryococcus* and the inflow of humus from the catchment caused a significant reduction in the penetration of light in the water column, and thus inhibited the development of life in the planktonic zone. The youngest sediments in Lake Suchar IV showed another change in the lake ecosystem. The smaller content of *Sphagnum* and the appearance of *Nymphaea*, as well as the presence of *Plumatella* specimens is indicative of declining dystrophic conditions. This situation is also reflected in the reduced content of littoral specimens and the arrival of Cladocera and diatom species living in the open water zone. Palynological analysis showed the presence of human indicators. It appears that the observed changes in the youngest sediments are most likely induced by human impact.

Our results demonstrate that dystrophy is a fairly unstable (disharmonic) state and is subject to fluctuations because of regional climatic and anthropogenic changes. The encroachment of vegetation on the water bodies may proceed from the shores toward the central part of a reservoir or from the water surface toward the bottom (formation of *Sphagnum* mats). The process is primarily determined by the type of catchment, i.e. catchment vegetation and the availability and the type of allochthonous material. The catchment area around Lake Suchar IV was dominated by forest of *Pinus*, so the supply of nutrients into the water body was insignificant. Researchers studying the history of Scandinavian boreal lakes also found large fluctuations during the transformations of oligotrophic lakes into dystrophic ones (Alhonen, 1987; Korsman *et al.*, 1994; Rantala *et al.*, 2015). They showed that the main cause of dystrophication of lakes were climate changes as a result of which humic acids were washed away from the drainage basin.

CONCLUSIONS

1. The results of the interdisciplinary sediment study introduced here showed that Lake Suchar IV has continuously developed from the Late Glacial to the present day.
2. The lake acquired its modern dystrophic nature at the beginning of the second half of the Atlantic period and was preceded by an unstable period of transition.
3. Given the biological and chemical composition and lithology of sediments, the development of the lake and its trophic state was affected mainly by regional factors such as climate and the conditions and quality of the drainage basin.
4. The harmonic development (oligo-, α-meso-, β-mesotrophic) was disturbed by an excessive influx of humic substances at the end of Atlantic period, the growth of *Sphagnum* and *Botryococcus* (especially in Subatlantic period), which resulted in the reduced light availability, thus changing the species composition of flora and fauna as well as pH of water, and consequently changing the trophic state into the dystrophic one.
5. The harmonic development of the lake and the increasing trophic conditions reflected regional climate changes. Warmer and colder periods were manifested through the species composition and frequency of phyto- and zooplankton.
6. During the disharmonic development period, the lake was dominated by acidophilous

species, indicative of low water pH and the trophic status at the level of dystrophy.

7. The transition between the harmonic and disharmonic development was preceded by a transitional period that was characterized by considerable fluctuations, especially in the phyto- and zooplankton structure.

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The first high resolution diatom record from Lake Balaton, Hungary in Central Europe

Krisztina Buczkó[1,2,*], Éva Ács[1], Katalin Báldi[3], Virág Pozderka[4], Mihály Braun[5], Keve Tihamér Kiss[1] and János Korponai[6,7]

[1] MTA Centre for Ecological Research, Danube Research Institute, 1113 Budapest, Karolina St 29, Hungary.
[2] Hungarian Natural History Museum, Department of Botany, 1088 Budapest, Baross St 13, Hungary.
[3] Eötvös Loránd University, Department of Physical & Applied Geology, 1117 Budapest, Pázmány Péter St 1/c, Budapest, Hungary.
[4] Eötvös Loránd University, Doctoral School of Environmental Sciences, 1117 Budapest, Pázmány Péter St 1/c, Budapest, Hungary.
[5] Isotope Climatology and Environmental Research Centre (ICER), Institute for Nuclear Research, Hungarian Academy of Sciences, 4026 Debrecen, Bem Square 18/C, Hungary.
[6] Department of Environmental Sciences, Sapientia Hungarian University of Transylvania, 400193 Cluj-Napoca, Calea Turzii str. 4., Romania.
[7] MTA - PE Limnoecology Research Group, 8200 Veszprém, Wartha Vince St 1. Hungary.

[*] Corresponding author: buczko.krisztina@okologia.mta.hu

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ABSTRACT

The first high resolution diatom record from Lake Balaton, Hungary in Central Europe

A high-resolution diatom record of the last 7500 years of Lake Balaton, a large, shallow calcareous lake in Central Europe is presented here. Diatom analyses were carried out on a 112 cm long core obtained from the Siófok Basin (eastern part of the lake) combined with loss-on-ignition, geochemical measurements and grain-size analysis. Altogether 140 diatom taxa were distinguished in this study; *Amphora pediculus*, *Cocconeis neothumensis*, *Fragilaria brevistriata* and *Karayevia clevei* were frequent and ubiquitous members of diatom assemblages. Fragilaroid taxa were dominant through the core in the shallow Lake Balaton, but episodes of higher ratios of planktic forms (*Lindavia balatonis*, *Aulacoseira granulata* and *Pantocsekiella ocellata*) before ca. 2000 cal yr BP, indicated high water level in the lake, and stronger fluctuation than exists today. The diatom-based reconstruction of lake level was in accordance with the geochemical and zoological records, proving the usability of siliceous remains in paleolimnological reconstructions in Lake Balaton, in spite of the unfavourable preservation conditions.

Key words: diatoms, geochemistry, Lake Balaton, lake level changes, shallow lake sediments

RESUMEN

El primer registro de diatomeas de alta resolución del lago Balaton, Hungría, en Europa Central

Aquí se presenta un registro de diatomeas de alta resolución del lago Balaton de los últimos 7500 años, un gran lago calcáreo poco profundo en Europa Central. Los análisis de diatomeas se llevaron a cabo en un testigo sedimentario de 112 cm de longitud obtenido en la cuenca Siófok (parte oriental del lago), combinado con pérdidas por ignición (LOI), mediciones geoquímicas y análisis de tamaño de grano. En este estudio se distinguieron 140 taxones de diatomeas; Amphora pediculus*,* Cocconeis neothumensis*,* Fragilaria brevistriata *y* Karayevia clevei *fueron miembros frecuentes y ubicuos de los conjuntos de diatomeas. Los taxones de Fragilaroides eran dominantes a través del testigo en el somero lago Balaton, pero los episodios de mayor proporción de formas planctónicas (*Lindavia balatonis*,* Aulacoseira granulata *y* Pantocsekiella ocellata*) antes de ca. 2000 calyr BP, indicaron un nivel de agua alto en el lago y una fluctuación más fuerte que la que existe hoy en día. La reconstrucción del nivel del lago basada en diatomeas estuvo de acuerdo con los registros geoquímicos y zoológicos,* *demostrando la utilidad de los restos silíceos en reconstrucciones paleolimnológicas en el lago Balaton, a pesar de las condiciones de preservación desfavorables.*

Palabras clave: *diatomeas, geoquímica, lago Balaton, cambios en el nivel del lago, sedimentos someros del lago*

INTRODUCTION

Lakes are complex ecosystems, playing vital roles in regulating the global hydrological and biogeochemical cycles and acting as important parts of the global biosphere (Romero-Viana *et al.*, 2009; Williamson *et al.*, 2009). Paleolimnology – a rapidly developing field of science – gathers information about the ontogeny of different kinds of waterbodies, mires, wetlands, ponds and lakes. Among these, shallow lakes are extremely sensitive to any stressors, even as they carry exceptionally high biological conservation values (Kearns *et al.*, 1996, Heinsalu *et al.*, 2007b). Shallow lakes and wetlands are important subjects of hydrological reconstruction, as they are more vulnerable than deep lakes to water level fluctuation. Even a small scale variation can cause considerable changes due to their shallowness. It is especially true for such a large and shallow lake as Lake Balaton, where the wind, combined with shallowness makes the lake prone to resedimentation, affecting benthic life (Korponai *et al.*, 2010).

Diatoms are unicellular algae; they are sensitive indicators of environmental changes. They have been successfully used to monitor disturbances, like eutrophication, changes to the physical and chemical properties of water, as well as fluctuations of water level (Stoermer & Smol 1999). However, though there is a wide consensus about the usefulness of diatoms in quantitative paleolimnological reconstruction (Stoermer & Smol 1999; Romero-Viana *et al.*, 2009; Heinsalu *et al.*, 2007a, 2007b), in Lake Balaton, no high-resolution diatom record has been published so far; therefore, the study presented here is entirely new.

Paleolimnological research on Lake Balaton

In spite of the intensive and continuous long-term neolimnological studies of Lake Balaton (Hatvani *et al.*, 2011, Istvánovics & Honti, 2018), the paleolimnological exploration of the lake is sparse and uneven, and is sometimes even questioned (WEB1 2018). There has been no comprehensive modern survey conducted on the entire lake, and only limited efforts were made for using multi-proxy approaches for paleoecological reconstruction concerning the history of the lake. In particular, the possibly uneven and dissimilar development of the different basins of Lake Balaton has been neglected.

Nevertheless, from the end of the nineteenth century, the lake and its surroundings have been intensively studied (Lóczy, 1916). It must be mentioned about this period that the first diatom record by J. Pantocsek (1913) presented a very low-resolution list with presence/absence data of a 4 meter long core from Siófok Basin.

In 1981, the Geological Institute of Hungary (MÁFI) initiated a comprehensive survey of the limnogeology of Lake Balaton. Altogether 33 boreholes were drilled into the Quaternary layers down to 10–12 m. The thickness of the Holocene mud was 5 meters on average, but it was widely varying from place to place, e.g. 10 meters in the western basin and less than 2 meters in the eastern end of the lake (Cserny & Nagy-Bodor 2000, Cserny 2002, Tullner & Cserny, 2003). These studies revealed that Lake Balaton is 15 000–17 000 years old, and its development started with the formation of several shallow water lakes. Buczkó *et al.* (2005) summarised the diatom data gathered by Márta Hajós working on 18 of these 33 cores. More than 350 taxa were listed in this compilation, but evaluation of data has never been completed. Shortcomings of the Márta Hajós dataset preclude it from being used in this study. No standard counting methods were followed (e.g. Battarbee, 1986), resulting in varying sample sizes, where the number of counted valves could range from less than ten to 1300 valves (Tó-29). Sometimes, even the counted valves of different slides were merged into one single entry in the database. Consequently, there is no simple statistical method that can be applied for the Márta Hajós dataset. Additionally, in these 33 cores, the organic content of samples was not measured, leaving us without this standard geochemical proxy.

More recently Korponai *et al.* (2010) made the first attempt to use multi-proxy methods in the ancient Lake Balaton where extended wetlands were recently reconstructed (Hatvani *et al.*, 2011). The site for coring was chosen in a formerly water covered area on the south-western part of the lake. Three different stages were established concerning water level on the basis of geochemical data, subfossil Cladocera and diatom remains in the sediments of the Zalavár Pond, a part of the Kis-Balaton wetland (Buczkó *et al.*, 2009; Korponai *et al.*, 2010). It should be emphasized that the coring site of Korponai *et al.* (2010) is beyond the present day open-water part of Lake Balaton, so the result of this multi-proxy study has its limitations concerning the history of the lake compared to the present coring site positioned centrally in a sedimentary basin which provides a sedimentary record embracing a longer time period.

Within the frame of the EuLakes project (WEB1 2018), there was an attempt to use high-resolution trophic reconstruction in the south-western basin of Lake Balaton. In conclusion, the applicability of diatom analyses was questioned for such a shallow, wind disturbed, calcareous lake as Lake Balaton (WEB1 2018).

The present study is, first of all, intended to test the applicability of diatoms as a paleolimnological proxy in the eastern basin of Lake Balaton. The goals of this work are twofold: first, to publish the first high-resolution diatom record from Lake Balaton with special attention to the preservation of small celled valves; secondly, to perform lake level reconstruction, using geochemistry, and subfossil diatom remains in the sediment. To achieve these goals, the Siófok Basin at the eastern end of Lake Balaton, characterized by high human pressure, but low direct input from the watershed, was chosen (Fig. 1.).

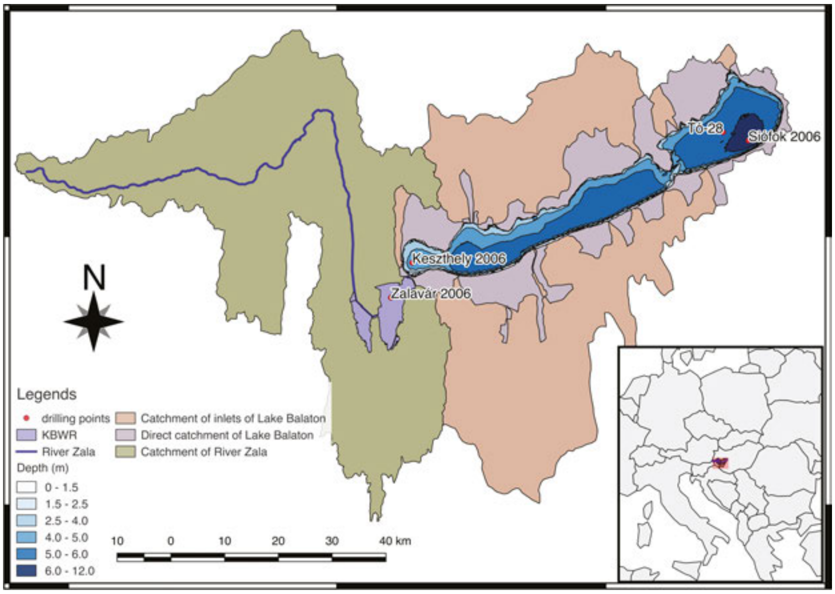

Figure 1. Lake Balaton and its catchment area. Note the four basins (Keszthely, Szigliget; Szemes, Siófok) and the difference between theirs catchment area size. Three cores were drilled in 2006 (Buczkó *et al.*, 2009, Korponai *et al.*, 2010). Tó-28 drilled within the MÁFI actual geological survey (Cserny, 2002, Rokob *et al.*, 2008). *El Lago Balaton y su área de influencia. Tenga en cuenta las cuatro cuencas (Keszthely, Szigliget, Szemes, Siófok) y la diferencia entre el tamaño de su área de influencia. Se perforaron tres testigos sedimentarios en 2006 (Buczkó* et al.*, 2009, Korponai* et al.*, 2010). Tó-28 perforado dentro de una verdadera investigación geológica MÁFI (Cserny. 2002, Rokob* et al.*, 2008).*

MATERIAL AND METHODS

Study site

Lake Balaton, the largest shallow lake in Central Europe, is one of the best studied lakes in the world (Entz & Sebestyén 1946, Istvánovics *et al.*, 2007, Istvánovics & Honti 2018). Its catchment area is 5175 km^2, with a major inflow, the Zala River in the west, with an average discharge of about 5–10 m^3/seg. The only outflow is the artificial Sió canal constructed in the 1860s connecting the lake to the Danube River (Fig. 1). The Zala River enters Lake Balaton through the Kis-Balaton Wetland, a large area acting as a natural filter for suspended material and nutrients that would otherwise be deposited in the lake (Fig. 1. Hatvani *et al.*, 2011). The lacustrine sediments are rich in carbonate (calcareous mud) increasing from south-west (Keszthely) to north-east (Siófok) (Cserny & Nagy-Bodor, 2000; Tullner & Cserny, 2003).

Field and laboratory methods

A sediment core (SIOFOK2006) of 112 cm length was obtained from the deepest, central part of the Siófok Basin in Lake Balaton (Fig. 1.) with a 5 cm diameter Livingston piston sampler in May 2006. Subsamples were taken at every second cm for siliceous algae, grain size and geochemical analyses. The procedure for grain size determination started with disaggregation by shaking wet samples for 24 hours before fractionating them in a settling column. For loss-on-ignition analysis, samples were weighed and heated at 550 ºC for 4 hours (Heiri *et al.*, 2001) and at 950 ºC for 2 hours. Trace element content was determined by ICP-OES - Thermo Iris Intrepid II XSP (Korponai *et al.*, 2010). For radiocarbon calibration of the core, macrophyte and charred plant remains were selected and (Table 1) analysed at the Laboratory of Environmental Studies, Institute of Nuclear Research, Hungarian Academy of Sciences in Debrecen. Dates were calibrated using the software CALIB REV5.0.0 (Stuiver *et al.*, 2005).

In order to analyse siliceous algae, sediment samples were prepared using standard digestion procedures (10 % hydrochloric acid, and 30 % H_2O_2 hot hydrogen-peroxide) (Battarbee, 1986), and at least 400 valves were counted in each sediment layer. For light microscope analysis, a LEICA DM LB2 was used (100 X HCX PLAN APO). Diatom identification followed the nomenclature of Hofmann *et al.* (2011) and Wetzel *et al.* (2015) for small celled naviculoids. Nomenclature was checked and updated according to AlgaeBase (Guiry & Guiry, 2016), accessed 2–25 April 2016, except genus *Pantocsekiella* where the concept of Ács *et al.* (2016) was followed. Stratigraphic zone boundaries were defined using CONISS on square-root transformed data without downweighting of rare taxa with the Psimpoll 4.27 program (Bennett, 2005). The significant number of diatom assemblage zones (DAZ) was assessed by a broken-stick model implemented in "Rioja" package (Juggins, 2012).

RESULTS

Physical properties of the core

Lithology, age, organic material content expressed in loss on ignition (LOI) at 550 ºC and at 950 ºC, Ca and Mg content, Fe and Mn content and their ratio according to the grain size distribu-

Table 1. Results of the ^{14}C dating measurements, SIOFOK2006 core; Lake Balaton. *Resultados de las dataciones con ^{14}C, testigo SIOFOK2006; Lago Balaton.*

Lab-code	Depth below core top (cm)	D13C (PDB) ± 0.2 [‰]	^{14}C year BP	Cal. year (2s) Cal yr BP	Mid-point of 2sigma calibrated age range
deb-13938	Siófok 59-64 cm	-0.53	3920 ± 50	4468 – 4516	4492
deb-13947	Siófok 104-109 cm	+1.05	6330 ± 60	7158 – 7421	7394

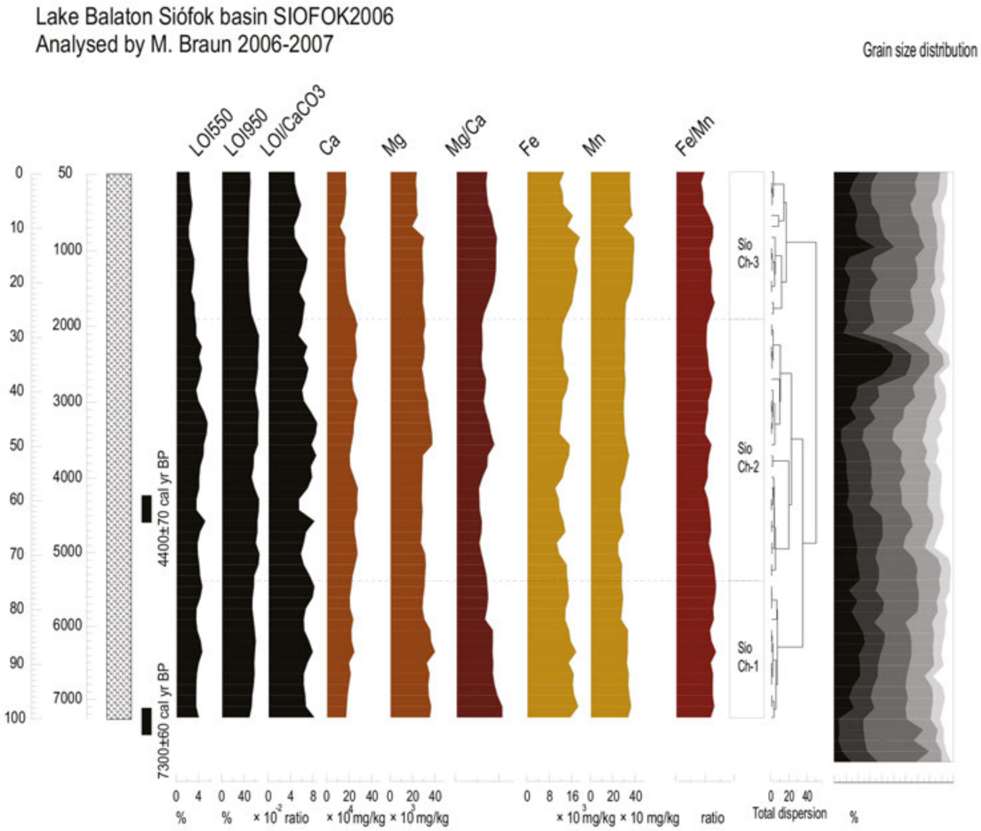

Figure 2. Physical properties of the core SIOFOK2006, Siófok Basin, Lake Balaton. Lithology, organic material content, main cations (Ca, Mg, Fe, Mn); Mg/Ca and Fe/Mn ratios are also plotted. Sio-Ch: local geochemical assemblage zones. Grain-size distribution, Sio-Gr: local grain size assemblage zones. *Propiedades físicas del testigo SIOFOK2006, Cuenca Siófok, Lago Balaton. Litología, contenido en materia orgánica, principales cationes (Ca, Mg, Fe, Mn); También se han representado las relaciones Mg/Ca y Fe/Mn. Sio-Ch: Zonas de agrupación geoquímica. Distribución granulométrica, Sio-Gr: Zonas granulométricas.*

tion are presented in Figure 2. Although the core SIOFOK2006 turned out to be quite uniform, homogeneous and unvaried in terms of lithology, and geochemistry, e.g. organic material content. Still, three zones could be distinguished.

Organic material content estimated by LOI_{550} is low, varying in a very narrow range (2-5 %), and shows some decrease at the top of the core. The LOI_{950} is high (40-65 %) and indicates three intervals. It is lowest at the top 30 cm. Higher values characterize the region between 30 and 75 cm, whereas below 75 cm has lower values. The topmost sample does not show high LOI values (LOI_{550} 2 % and LOI_{950} 46 %) indicating continuously disturbed, mixed sediments, preventing rich benthic vegetation on the bottom of the lake.

Five zones (Sio-Gr1–Sio-Gr5) were distinguished using the grain size distribution (Fig. 2). Generally, the core is silty, except for a sand fraction peaking between 28 and 38 cm, with grains of calcareous aggregates, most likely formed autogenetically as part of diagenetic processes.

The two successful measurements of radiocarbon dates are presented in Table 1. A linear sedimentation rate model was applied, establishing the linear relationship between age and depth. The high carbonate and low organic content complicated the radiocarbon dating of the core, making the applied time frame less than ideal concerning reliability. The studied 112 cm long core spans ca. 7500 years.

Table 2. List of diatom taxa in SIOFOK2006 core was found > 5 % in relative abundance in samples (%). The first column gives the taxon relative abundance in the whole core, while the second column show the number of occurrences in the 53 studied samples. *La lista de taxones de diatomeas en el testigo SIOFOK2006 fue > 5% en abundancia relativa en las muestras (%). La primera columna da la abundancia relativa del taxón en todo el testigo, mientras que en la segunda columna muestra el número de ocurrencias en las 53 muestras estudiadas.*

List of taxa in Siofók Basin of lake Balaton (V. Pozderka 2015-2016)	Relative abundance	Frequency
Amphora copulata (Kützing) Schoeman & R.E.M.Archibald	0.83	42
Amphora pediculus (Kützing) Grunow ex A.Schmidt	7.65	54
Aneumastus minor Lange-Bertalot	0.2	23
Aneumastus tuscula (Ehrenberg) D.G.Mann & A.J.Stickle in Round, Crawford & D.G.Mann	0.11	15
Aulacoseira granulata (Ehrenberg) Simonsen	6.95	54
Belonastrum berolinensis (Lemmermann) Round & Maidana	2.73	51
Biremis zachariasii (Reichelt) Edlund, N.A.Andresen & Soninkhisig	0.28	23
Caloneis schumanniana (Grunow) Cleve	0.15	5
Campylodiscus hibernicus Ehrenberg	0.12	14
Cavinula jaernefeltii (Hustedt) D.G.Mann & A.J.Stickle in Round. Crawford & D.G.Mann	0.1	6
Cavinula scutelloides (W.Smith) Lange-Bertalot in Lange-Bertalot & Metzeltin	0.46	39
Cocconeis neodiminuta Krammer	2.2	46
Cocconeis neothumensis Krammer	2.65	54
Cymbopleura inaequalis (Ehrenberg) Krammer	0.13	5
Diploneis elliptica (Kützing) Cleve	0.12	9
Diploneis oblongella (Nägeli ex Kützing) Cleve-Euler	0.44	16
Diploneis oculata (Brébisson) Cleve	0.29	11
Diploneis pseudovalis Hustedt	0.96	37
Fallacia subhamulata (Grunow) D.G.Mann in Round. R.M.Crawford & D.G.Mann	0.22	17
Fragilaria brevistriata Grunow in van Heurck	21.06	54
Fragilaria construens (Ehrenberg) Grunow	3.11	37
Gyrosigma acuminatum (Kützing) Rabenhorst	1.27	45
Hippodonta costulata (Grunow) Lange-Bertalot. Metzeltin & Witkowski	1.88	41
Karayevia clevei (Grunow) Round & Bukhtiyarova	3.24	53
Lindavia balatonis (Pantocsek) T.Nakov *et al.*,	0.96	25
Navicula cryptotenella Lange-Bertalot in Krammer & Lange-Bertalot	0.15	11
Navicula menisculus Schumann	0.14	13
Navicula seminuloides Hustedt	9.34	52
Neidium binode (Ehrenberg) Hustedt	0.24	23
Neidium dubium (Ehenberg) Cleve	0.15	21
Neidium iridis (Ehrenberg) Cleve	0.1	5

Cont.

Table 2. (cont.)

Nitzschia recta Hantzsch ex Rabenhorst	0.1	10
Pantocsekiella ocellata (Pantocsek) Kiss & Ács	1.34	43
Parlibellus crucicula (W.Smith) Witkowski, Lange-Bertalot & Metzeltin	0.16	21
Pinnularia balatonis (Pantocsek) Mills	0.13	15
Pinnularia viridis (Nitzsch) Ehrenberg	0.17	12
Placoneis pseudanglica E.J.Cox	0.23	27
Pseudostaurosira parasitica (W.Smith) Morales	0.86	39
Sellaphora nigri (De Notaris) C.E.Wetzel & L.Ector in Wetzel *et al.*,	1.36	30
Sellaphora rotunda (Hustedt) C.E.Wetzel. L.Ector. B.Van de Vijver. P.Compère & D.G.Mann	0.96	26
Sellaphora utermoehlii (Hustedt) C.E.Wetzel & D.G.Mann in Wetzel *et al.*,	7.62	50
Sellaphora verecundiae Lange-Bertalot	0.17	8
Stauroneis anceps Ehrenberg	0.3	30
Staurosira tabellaria (W.Smith) Leuduger-Fortmorel	4.34	43
Staurosirella pinnata (Ehrenberg) D.M.Williams & Round	11.82	55
Surirella amphioxys W.Smith	0.14	19
Tryblionella angustata W.Smith	0.1	13

Biological remains

Diatoms

Altogether about 140 taxa were distinguished during the study (Table 2); *Amphora pediculus*, *Cocconeis neothumensis*, *Fragilaria brevistriata*, and *Karayevia clevei* were not just abundant, but ever present members of the diatom assemblage. The small fragilaroid taxa (*Staurosirella pinnata*, *Staurosira tabellaria*) and small naviculoid forms like *Navicula seminuloides* and *Sellaphora nigri*, *S. rotunda* and *S. utermoehlii* were found abundant in some layers. The genus Amphora (*A. pediculus* and *A. copulata*) was also often present in the samples. Biofilm constituent diatoms growing on macrophytes are also often registered, e.g. *Cocconeis neothumensis* and *C. neodiminuta*. Three planktic taxa all belonging to centric diatoms, *Aulacoseira granulata*, *Lindavia balatonis* and *Pantocsekiella ocellata* significantly contribute to the assemblages. Two diatom species, *Karayevia clevei* and *Belonastrum berolinensis* are believed to be characteristic to Lake Balaton and were common in the majority of samples. The presence of the endemic diatom *Pinnularia balatonis* Pantocsek can also be detected along the entire core.

Diatom assemblage zones – succession of Siófok Basin

Relative frequency changes of the most common taxa throughout the sequence are shown in Figure 3. In the diatom dataset, six statistically significant diatom assemblages zones (DAZ) were distinguished. DAZs are presented below according to depth:

SIO-DAZ-1 112–104 cm – Small, but robust fragilaroid taxa (mainly *Staurosira pinnata*) with the motile *Caloneis schumanniana* and *Neidium iridis* indicate an unstable and changing environment in the lower studied layer (Stenger-Kovács *et al.*, 2018).

SIO-DAZ-2 104–82 cm – *Fragilaria brevistriata*, *Aulacoseira granulata* with small celled naviculoids (like *Navicula seminuloides* and *Sellaphora utermoehlii*) with the slightly motile

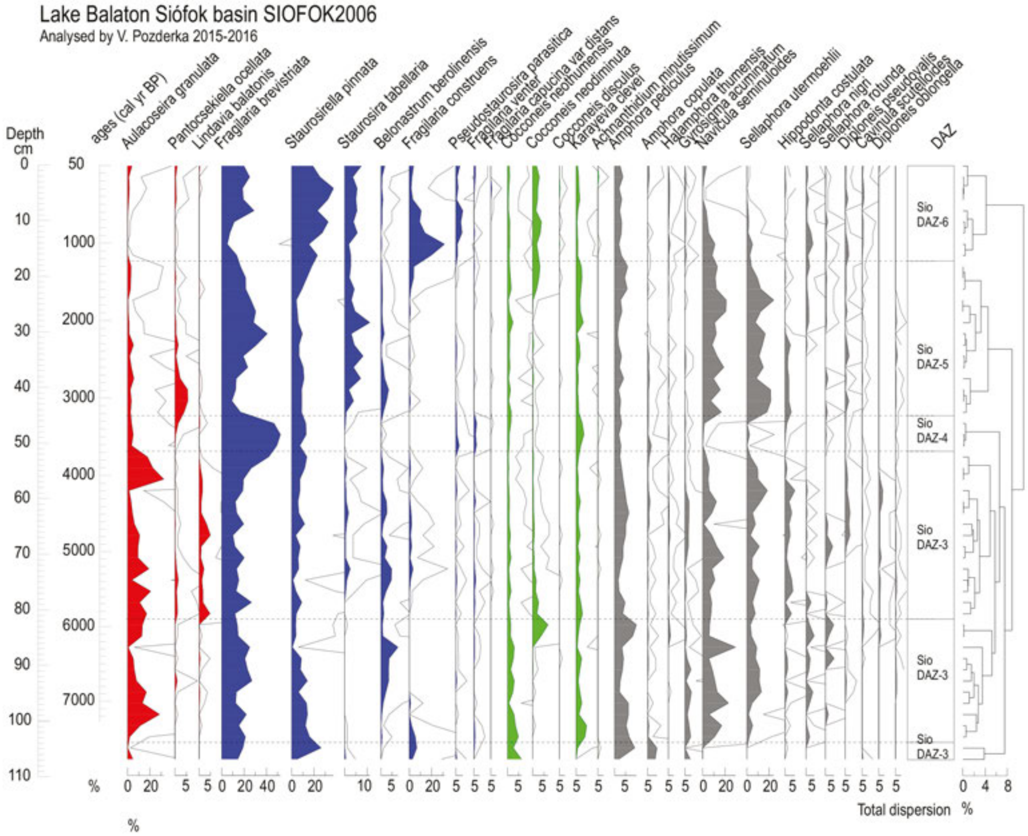

Figure 3. Relative abundance diagram of important diatom taxa in SIOFOK2006 core. Plankton taxa are marked by red; fragilaroid diatoms are blue, while the most typical periphytic taxa are green on the diagram. Lines without filling are exaggerated (10x) Sio-DAZ: diatom assemblage zone. *Diagrama de abundancia relativa de importantes taxones de diatomeas en el testigo SIOFOK2006. Los taxones planctónicos están marcados con rojo; las diatomeas fragilaroid son azules, mientras que los taxones perifíticos más típicos son verdes en el diagrama. Las líneas sin relleno están exageradas (10x) Sio-DAZ: zona de agrupación de diatomeas.*

Amphora pediculus are abundant in this zone. *Cocconeis* taxa are also present. The whole community points to rather diverse habitats in the basin referring to makrophytes. The species *Belonastrum beroliensis* is a not too common, but an often reported diatom in Lake Balaton, showing a minor maximum in this zone.

SIO-DAZ-3 82–54 cm – The zone is characterized by similar assemblage as the one below, except for the species *Lindavia balatonis* becoming characteristic for this zone, indicating a rise in water level. The relatively high portion of *Aulacoseira granulata* confirms the reconstruction of high water level. The end of this zone shows a peak of *A. granulata*.

SIO-DAZ-4 54–46 cm – This very short zone is clearly dominated by the benthic, commonly chain-forming *Fragilaria brevistriata*.

SIO-DAZ-5 46–20 cm – Though the relative abundance of fragilaroid taxa is high, just as before and throughout the entire core, the small celled naviculoids like *Navicula seminuloides*, *Sellaphora nigri*, *S. rotunda* and *S. uthermoehlii* are also characteristic for the diatom assemblage. The ratio of planktic forms is very low. *Staurosira tabellaria*, one of the most characteristic taxon of Lake Balaton (Ács *et al.*, 2009) has become abundant in this zone, confirming its continuing importance to this day.

SIO-DAZ-6 20–0 cm – The upper zone is clearly characterized by fragilaroids, of mainly the small celled type, but also the heavily silici-

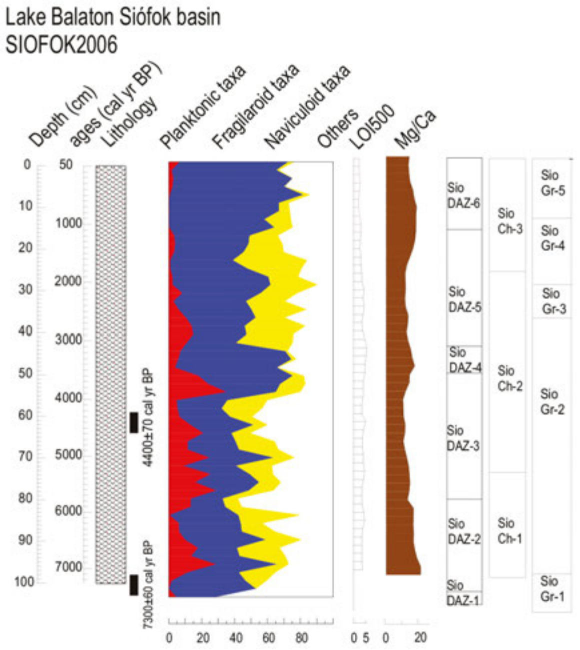

Figure 4. Lithology, Ca/Mg ratio, organic material content (LOI 550), and zone boundaries of the studied proxies in SIOFOK2006 core. Note that the diatom, geochemical and grain size analysis were carried out not on the same length of core. *Litología, relación Ca/Mg, contenido de materia orgánica (LOI 550) y límites de zona de los proxies estudiados en el núcleo SIOFOK2006. Téngase en cuenta que el análisis de diatomeas, geoquímica y tamaño de grano no se llevó a cabo en la misma longitud del testigo.*

fied *Staurosirella pinnata* is present. Increasing abundance of the epiphytic *Cocconeis neodiminuta* species is detected in this zone.

Lake level fluctuation is reconstructed based on diatom proxies reflecting the changed proportion of taxa characterized by different life forms in the assemblage. The assemblages dominated by planktic/tychoplanktic diatoms like *Aulacoseira*, *Lindavia*, *Pantocsekiella* and *Belonastrum* taxa indicate high lake levels (Fig. 4.). Fragilaroid taxa were found dominant throughout the core in the Siófok Basin due to the shallowness of Lake Balaton, but the increased ratio of planktic forms implied higher lake levels in the past. *Lindavia balatonis* was the dominant planktic species between 82–56 cm. In these layers the species *Aulacoseira granulata* was also frequently observed, exhibiting a peak between 56–52 cm. At 46–38 cm the planktic *Pantocsekiella ocellata* reached higher abundance. According to this dataset Lake Balaton had a rather high, but fluctuating lake level before 2000 cal yr BP. Between 46–22 cm some small celled (< 10 µm) diatom taxa prevailed, suggesting a decreasing lake level. The species *Navicula seminuloides*, *Sellaphora nigri*, *S. rotunda*, and *S. utermoehlii* are also worthwhile to mention among the taxa that have been identified so far in the assemblages.

DISCUSSION

It is a well-known fact that the thickness of Holocene sediment shows a gradient along the long axis of the Lake Balaton, (e.g. Müller & Wagner, 1978; Tullner & Cserny, 2003; Visnovits, 2015): Holocene sediments can reach 10 m in the western part of the lake, where the river Zala with a rather extended catchment area enters, while it reaches only ca. 2 m in the central part of the eastern basin of the lake. Moreover, at some particular places along the lakeshore the Holocene sediments can be altogether missing (Visnovitz, 2015, Visnovitz *et al.*, 2015). This highly variable sediment thickness of the lake can make paleolimnological interpretation difficult.

Physical properties of SIOFOK2006 core

The relationships between LOI at 550 °C (LOI$_{550}$) and organic carbon content and between LOI at 950 °C (LOI$_{950}$) and inorganic carbon content are currently accepted as a standard proxy for organic matters (Santisteban *et al.*, 2004). Our results (very low LOI$_{550}$ and high LOI$_{950}$) are consistent with these main features, the ratio of biotas and calcareous mud of Lake Balaton.

The grain size distribution of the lake is more about diagenetic processes than the energy of water, as the sand fraction sized grains are autochthonous calcareous aggregates.

In the frame of EuLakes project four lakes were cored (two of them were shallow ones); and only Lake Balaton was found unsuitable for reconstruction of historical trophic state of the lake (https://www.eulakes-model.eu/outputs/ecological-evolution/index.html). Undoubtedly, the shallowness and the strong influence of wind at Lake Balaton makes the sediment record of the lake far from an ideal subject for high-resolution paleo-ecological analyses, but our data and results obtained from SIOFOK2006 core show adequate preservation where the small, fine valves of diatoms were preserved in the mud (Kiss *et al.*, 1999).

Taxonomic remarks

Although, Lake Balaton is one of the best studied lakes in the world, the diatom flora received less attention, except in the initial pioneering phase of scientific exploration in the 19th century (Lóczy 1916, Pantocsek 1901, 1913). Unfortunately, there is no recent overview, or checklist available of the diatom flora, only a few compilations were published at the middle of the last century (Szemes 1957, Tamás 1965).

The diatom flora of Lake Balaton is unique, with some endemic species like *Pinnularia balatonis*, a ubiquitous member of the diatom assemblage throughout the core. The taxonomy of fragilaroids, exhibiting huge variability with overlapping forms has been a challenge in Lake Balaton (Ács *et al.*, 2009), especially when comparative analysis is required. It's worthwhile to mention the uncertainty of the species-level determination of *Aulacoseira* as *A.* aff. *granulata* in Lake Balaton. As previous paleolimnological studies of Lake Balaton revealed, the very rich diatom populations from the deeper sediment layers might help to resolve taxonomic problems. For example, the *Aulacoseira* taxa are dominant along the long axis of the lake as subfossils, a finding that helps to resolve the taxonomic difficulties concerning the genus in the lake. The species *Belonastrum berolinensis*, although a wide spread taxon (Round & Maidana 2001), is generally rare in Hungary, but here in Lake Balaton, it is regarded as a typical, frequent element of the flora.

The above mentioned taxonomical uncertainties have been known for a long time, but this study on SIOFOK2006 core offers new taxonomic challenges concerning the small naviculoid species of Lake Balaton. Among the small naviculoid (< 10 µm), species like *Navicula seminuloides*, *Sellaphora rotunda*, *S.nigrii* and *S. utermoehlii* have been so far successfully identified from the assemblages. The accurate identification requiring the comparison with type materials is in progress, but still remains a challenge (as in Kiss *et al.*, 2007).

Diatom-based reconstruction on SIOFOK2006 core

Changes in the planktic/benthic ratio of diatom assemblages indicate water level fluctuation (Heinsalu *et al.*, 2007a, Korponai *et al.*, 2010). In spite of the low organic material content (LOI < 5 %), the samples prepared directly for siliceous microfossil analysis from the Holocene lacustrine sediments obtained in the Siófok Basin of Lake Balaton were suitable for diatom analysis.

Changes of water depth: Wind disturbed, very shallow water, without, or with only sporadic macrophytes can be reconstructed on the basis of the diatom assemblages from 7500 cal yr BP. The higher lake level in the Holocene was reconstructed for the period 7500-2000 cal yr BP. Gradually decreasing water depth can be inferred from 2000 cal yr BP onward.

Several studies demonstrated uneven and mosaic hydrological changes from the entire area of the historical Lake Balaton (e.g. Cserny *et al.*, (1991), Jakab *et al.*, 2005, Rokob *et al.*, 2008, Korponai *et al.*, 2010). A multiproxy, non-diatom analysis was carried out in the north-western basin of the lake reconstructing the paleoenvironment (Jakab *et al.*, 2005). This study suggested that from 5200 cal yr BP the water depth increased in the studied area. This is in line with our findings in the Siófok Basin at the south-east end of the lake. If the same phenomenon is observed in two opposite basins of the lake, then these data suggest that the lake level increase affected the entire lake and was in all probability climate-driven. In the last 2000 year history of the lake, the decreasing lake level can be due to human-induced pressure.

Mainly alkalophilous or indifferent diatom species were recorded along the sequence, indicating that there was no significant change in the water chemistry. Due to taxonomical uncertainties in Lake Balaton, and the lack of local training sets for paleoecological reconstruction, the calculation of quantitative phosphorous and pH for the studied sequence is considered premature, but it could be an urgent task for better understanding the current ongoing processes in the lake (Korponai *et al.*, 2011). However, similar diatom records (the dominance of *Aulacoseira* taxa with small-sized fragilaroids) are often reported from large shallow lakes throughout the Holocene in Europe, maybe it is a typical assemblage for natural large lakes (Heinsalu *et al.*, 2007a).

It is well-known that the Zala river in the south-west end of the lake is the major source of inflow. The nutrient load arriving with this river controls many environmental factors in a south-west/north-east gradient along the longitudinal axis of the lake (e.g. Müller & Wagner, 1978; Tompa *et al.*, 2014), and seemingly, the preservation potential of silica in the sediment essential for the diatom record, might change from location to location also. Another high resolution study carried out in the lake is the EuLakes project (WEB1 2018), with sampling sites in the Keszthely Basin, which is exactly at the opposite end of Lake Balaton compared to the present site. Two ca. 70 cm long cores were collected at a depth of ca. 3 m. Even though the Keszthely Basin is a sub-basin of Lake Balaton receiving the highest nutrient load from the nearby Zala river during the past few decades, it is still characterised by high trophic level. However, their observation of "no diatom counts could be performed in the cleaned and mounted sub-samples due to the extremely scarce number of frustules and to their pronounced erosion. Only a few among the most robust and highly silicified taxa, could be identified in the observed samples. Planktic taxa are almost completely missing, with the exception of very rare specimens of *Cyclotella ocellata* Pantocsek (*Pantocsekiella ocellata* in this study) and *C. radiosa* (Grunow) Lemmermann / *balatonis* Pantocsek, (*Lindavia radiosa* and *L. balatonis* in this study) while the most represented benthic form is *Fragilaria martyi* (Hèribaud) Lange-Bertalot" is of limited value concerning the entire lake. The conclusion of the EuLakes project that "sediment diatoms of Lake Balaton are not representative of the living community, as a result of selective diatom elimination. The highly mineral and perturbed sediments of Lake Balaton likely represent a very aggressive environment for the most slender and delicate benthic and planktic taxa" can be easily refuted by the reasonably well preserved diatoms found in the Siófok Basin as presented here.

CONCLUSIONS

Several earlier studies demonstrated uneven and mosaic hydrological changes of the entire area of the historical Lake Balaton during the late glacial and Holocene times.

The use of diatoms in the paleoecological reconstruction of Lake Balaton has been questioned based on several less successful attempts conducted in the last decades. However, the SIOFOK2006 core yielded exploitable diatom flora for paleoenvironmental reconstruction.

Based on the diatom assemblages of the SIOFOK2006 core the following paleoenvironment can be reconstructed for the Siófok Basin of Lake Balaton, a wind disturbed, very shallow water without, or with only sporadic macrophytes living at the bottom of the lake. This environment prevailed from the basal layers of the core (from ca. 7500 cal yr BP) to the present, while gradually decreasing water depth can be inferred from 2000 cal yr BP onward.

Finding a high ratio of small celled naviculoid forms in the upper part of this sequence is unique, as no other earlier studied cores contained these forms in such high abundance.

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Taxonomic, functional and phylogenetic diversity: how subfossil cladocerans mirror contemporary community for ecosystem functioning: a comparative study in two oxbows

Korponai, János[1,2,*], Mihály Braun[3], László Forró[4], István Gyulai[5], Csilla Kövér[6], Judit Nédli[4], István Urák[2] and Krisztina Buczkó[7,8]

[1] MTA - PE Limnoecology Research Group, 8200 Veszprém, Wartha Vince u. 1, Hungary.
[2] Department of Environmental Sciences, Sapientia Hungarian University of Transylvania, 400193 Cluj-Napoca, Calea Turzii str. 4., Romania.
[3] Isotope Climatology and Environmental Research Centre (ICER), Institute for Nuclear Research, Hungarian Academy of Sciences, 4026 Debrecen, Bem tér 18/C., Hungary.
[4] Department of Zoology, Hungarian Natural History Museum, H-1088 Budapest, Baross u. 13. Hungary.
[5] Department of Hydrobiology, University of Debrecen, H-4032. Debrecen, Egyetem tér 1., Hungary.
[6] Food and Wine Research Center, Eszterházy Károly University, H-3300 Eger, Leányka út 6., Hungary.
[7] MTA Centre for Ecological Research, Danube Research Institute, 1113 Budapest, Karolina út. 29, Hungary.
[8] Department of Botany, Hungarian Natural History Museum, 1088 Budapest, Könyves Kálmán krt. 40., Hungary.

[*] Corresponding author: korponai.janos@iif.hu

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ABSTRACT

Taxonomic, functional and phylogenetic diversity: how subfossil cladocerans mirror contemporary community for ecosystem functioning: a comparative study in two oxbows

Biases of taxa diversity and traits of source (active) and subfossil cladoceran communities with phylogenetic and functional approach were studied in two oxbows of the River Tisza, Hungary. Subfossil cladoceran communities were found to have suffered species loss due to both the degree of heterogeneity in the lake and taphonomical processes. The remains of heavily chitinized species are those most commonly found in the sediment, and therefore bias communities reconstructed on this basis. These biases are mostly caused by: (1) parts of soft bodied filterers (Daphniidae, Moinidae, Sididae (*Diaphanosoma*)), which are underrepresented in the subfossil remains of cladocerans, and (2) the remains of species that are difficult to collect and, so, underrepresented or absent in contemporary samples; this may have a strong effect on diversity indices. In spite of such biases, the paleocommunity does reflect the functioning of the ecosystem. The subfossil cladoceran community in the Morotva-tó (MT) corresponded to low fish densities and the high macrophyte coverage, indicating weaker top-down forces in this oxbow. The phylogenetic and functional approach to subfossil cladocerans may be useful in paleolimnological reconstruction, since phylogenetic and trait-based diversity indices correctly predicted top-down regulation as a function of the ecosystem.

Key words: active and subfossil Cladocera; oxbow; paleocommunity; trophic status; paleolimnology

RESUMEN

Sesgos en la diversidad taxonómica, funcional y filogenética de las comunidades vivas y subfósiles de cladóceros

La diversidad de taxones y rasgos de las comunidades de cladóceros actuales (activa) y subfósiles (sedimentada) fueron estudiadas en dos galachos del río Tisza, Hungría. Se investigaron los sesgos entre las poblaciones actuales de cladóceros (comunidad de origen) y la comunidad de cladóceros subfósiles. Estas comunidades subfósiles mostraron una pérdida de especies debido a procesos tafonómicos. Los restos de las especies fuertemente quitinizadas se encuentran más comúnmente *en el sedimento, produciendo sesgos en las comunidades reconstruidas. Los sesgos son causados principalmente por, (1) las partes de filtradores de cuerpo blando (Daphniidae, Moinidae, Sididae (*Diaphanosoma*)) que están subrepresentados entre los restos subfósiles de cladóceros, y (2) los restos de especies que son difíciles de recolectar y están subrepresentadas o ausentes en las muestras actuales, y que pueden tener un fuerte efecto sobre los índices de diversidad. A pesar de los sesgos, la paleocomunidad refleja el funcionamiento del ecosistema. La comunidad de cladóceros subfósiles en la laguna MT reflejó una baja densidad de peces, y su alta cobertura de macrófitos indicó el bajo estado trófico de la laguna fluvial. El enfoque funcional en el estudio de cladóceros subfósiles puede ser útil en la reconstrucción paleolimnológica, ya que los índices de diversidad basados en estos rasgos podrían ser mejores indicadores el funcionamiento de los ecosistemas.*

Palabras clave: *cladóceros subfósiles; galacho; paleocomunidad; estado trófico; paleolimnología*

INTRODUCTION

Multifactor stressors force rapid changes in most ecosystems, and this calls for the intensification of biodiversity studies. Traditionally, biodiversity studies have been based on the number of species within a given ecosystem, and to a lesser extent on the distribution of individuals within a given species in an assemblage (Gaston, 2000; Hamilton, 2005). The majority of studies have focused on common taxonomic indices such as richness and evenness (Hill, 1973). Taxonomic diversity has been used to estimate biodiversity (α-, β-, and γ-diversity Whittaker, 1972) and spatial and temporal changes in relation to environmental or anthropogenic factors (e.g. Zhuravlev & Naimark, 2005; Blarquez *et al.*, 2014). Although this is a well-established approach, it ultimately provides little information on how changes in biodiversity (community composition) affect the functioning of an ecosystem. The correct identification of this is crucial in the case of taxon-based indices, but the biological and ecological role of the species might be of even greater importance in the functioning of an ecosystem (Walker, 1992). At the species- and ecosystem-levels, the identity of species and ecosystem functioning is the result of evolutionary processes and adaptation to given environmental conditions; therefore, the ecosystem functioning may be characterized by the composition of the functional traits of organisms. A functional trait may be defined as a characteristic of an organism relevant to its response to the environment, and thus, to its effects on the ecosystem functioning (Díaz & Cabido, 2001). Using zooplankton, Thompson *et al.* (2015) showed that phylogenetic and functional diversity better predicted ecosystem functioning than species richness. Phylogenetic diversity (PD) is used as a proxy for functional diversity (FD). The relationship between PD and FD is that evolutionary diversification has generated trait diversification, which may result in greater niche complementarity (Flynn *et al.*, 2011).

Zooplankton occupy a key position in trophic structure and have been used to understand the functioning of aquatic ecosystems (Jeppesen *et al.*, 1997, 2000, 2002, 2011; Iglesias *et al.*, 2011). The taxonomical and functional structure of zooplankton assemblages is influenced by both bottom-up and top-down forces. The nutrient enrichment of lakes fuels the phytoplankton growth, supplying zooplankton with food resources; therefore, at first sight, it seems to increase the biomass of zooplankton. However, to a great extent the relationship depends on the size distribution of the zooplankton. Large zooplankton species such as daphniids are effective filterers, hence their dominance can be expected in zooplankton assemblages in a phytoplankton-rich environment. At the same time, large bodied zooplankters are perfect prey for vertebrate predators; consequently, stocks of them will depend on their various voracious size-selective predators (Confer & Blades, 1975; Dodson, 1979). In this sense, zooplankton biomass as a function of an ecosystem may be characterized by the composition of zooplankton species that are effective filterers and have a good predator avoidance strategy. Zooplankton species differ in their trait properties (i.e. body size, clearance rate, habitat preference, feeding strategies, predator defense strategies etc.; see Barnett *et al.*, 2007; Pomerleau *et al.*, 2015; Gianuca *et al.*, 2017; Rizo *et al.*, 2017). Different ecosystem functions can be best predicted by different indices, depending upon different aspects of diversity. Thompson *et al.* (2015) found that zooplankton biomass is best predicted by zooplankton trait-based functional richness, while phytoplankton abundance, as result of the top-down effect of zooplankton, is best predicted by zooplankton phylogenetic diversity. Gianuca *et al.* (2016) clarified the role of body size in providing strong predictions of the top-down control of cladocerans.

Long-term changes in ecosystem structure and functioning can be easily observed in paleolimnological studies due the long time-span of such studies, which can range from decades to millennia (Smol, 2002; Nevalainen *et al.*, 2015; Nevalainen & Luoto, 2017). Remains of Cladocera are common in lake sediments and have been widely used in paleolimnological reconstructions (Korhola & Rautio, 2001, Smol, 2002). Cladocerans occupy a central position in the zooplankton community since the clearance rates of cladocerans are the highest of any zooplankton (Barnett *et al.*, 2007), resulting in a pronounced top-down effect on phytoplankton, and their becoming a vulnerable prey to their predators (top-down effect on Cladocera).

Biases can be found between the active population of cladocerans and the reconstructed community, based on its subfossil remains in the sediment (Kattel *et al.*, 2007; Nykänen *et al.*, 2009). These biases result mostly from the differential preservation of species, as well as those biases likely to be present in a central lake core, which are attributable to transport processes from littoral source communities. In spite of the biases, several studies have also demonstrated similarities between the contemporary community and sedimented assemblages (Jeppesen *et al.*, 2000; Davidson *et al.*, 2007). Moreover, Davidson *et al.* (2007) found that the forces that act on cladoceran communities (e.g. macrophytes and fish) were essentially the same for contemporary and subfossil assemblages.

In paleolimnological reconstruction, it is very important to know about biases between contemporary and paleocommunities. An assessment of the degree of biodiversity present in lakes requires large-scale, intensive sampling campaigns, due to their heterogeneous microhabitat structures. In paleolimnological studies, in contrast to neolimnology, generally a single core is used to obtain biological proxies. Moreover, changes in species matrices (biodiversity) of this single core are used for reconstruction of environmental changes. Lakes have complex biota structures due to in-lake heterogeneity of microhabitats. It is especially valid for shallow lakes because their in-lake heterogeneity may be higher due to their extended littoral zones. Therefore, large biases may be found in the structure of cladoceran communities in paleolimnological

Table 1. Characteristics of the oxbows. *Características de los galachos.*

	MT	Marótzugi-Holt-Tisza
GPS	N 48.160748°; E 21.477610°	N 48.175611; E 21.612306,
type	natural	Artificial; cut off in 1860
Length (m)	800	1800
Average width (m)	63	60
Area (ha)	5	11
Av. Depth (m)	1.30	1.20
municipality	Tímár	Gávavencsellő
Sluice to River Tisza	no	no

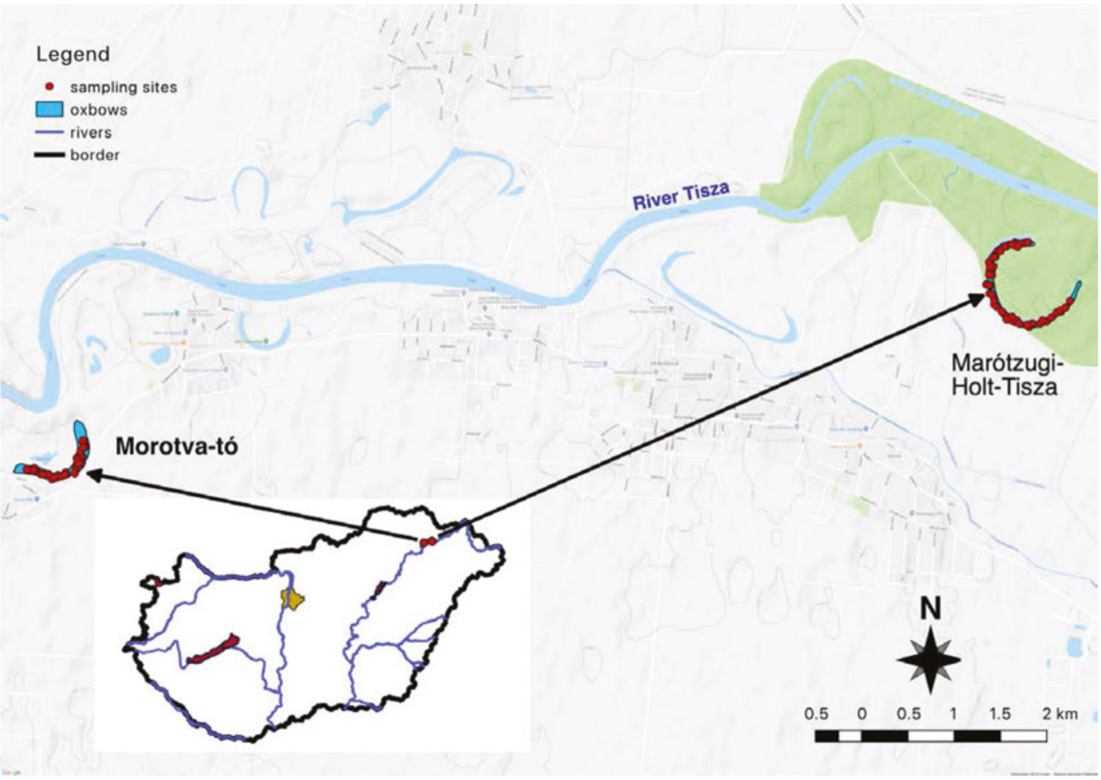

Figure 1. Map of the investigated oxbows. (Points on the maps of the oxbows represent sampling points in the course of fieldwork). *Mapa del galacho estudiado. (Los puntos representan los puntos de muestro).*

reconstructions of shallow lakes. Bias may originate in the differencing taphonomy of cladoceran species. Macrophyte beds favor the development of dense phytophilous Daphniidae populations (e.i. *Simocephalus* sp., and *Ceriodaphnia* sp.), while large populations of *Moina* sp. and *Diaphanosoma* sp. may be found in open waters. The weak preservation of their remains in the sediment tends to lead to the underestimation of their true proportion in the biodiversity of the lake. These biases have obvious effects on lake biodiversity assessment. Another factor is that, in spite of widespread intensive sampling efforts, only a part of benthic cladoceran species are retrieved, while the larger part of benthic species remain invisible to researchers, thus precluding any assessment of their role in biodiversity. In this pilot study the results of the intensive sampling of active cladocerans and subfossil remains of a single site in biodiversity assessments are compared.

For this pilot study two shallow oxbow lakes were chosen near the River Tisza, with the following aims: (1) to estimate how large a bias exists between contemporary and subfossil communities in these shallow lakes; (2) to determine the bias effect on the estimation of biodiversity indices; (3) to study how well the functional diversity of a paleocommunity reflects the functioning of an ecosystem, as derived from source communities.

MATERIAL AND METHODS

Study area

The River Tisza is the second largest river in Hungary. It drains an area of about 156 087 km^2 and has a length of 1419 km in Hungary. Its annual mean discharge is 792 m^3/s, and it contributes about 14 % of the Danube's total runoff

(Sommerwerk *et al.*, 2009). It flows through the Great Hungarian Plain. Since plains can cause a river to flow slowly, the Tisza used to follow a path with many curves and turns, which led to many large floods in the area. The regulation of the Tisza was carried out in the 19th century. In the 1860s, during the large river regulation program, 112 meanders were cut (Lászlóffy, 1982), shortening the river length by 474 km, and forming a number of artificial oxbow lakes. Prior to regulation, however, oxbow lakes also formed naturally. Oxbow lakes may be divided into two categories according to their location: un-protected oxbow lakes are situated between the levees and the river, and protected ones can be found on the far side of the dykes. Since oxbows are mostly supplied by water during the flooding of the Tisza, many of them have filled up and subsequently dried out. Although the artificial meanders are much younger than the naturally formed ones, most of them have attained a similar ecological status as the older, natural oxbows (Pálfai, 2003).

Two oxbow lakes, Morotva-tó (MT) and the Marótzugi-Holt-Tisza (MHT), were selected for living and subfossil cladoceran analyses. Both lakes are small; Morotva-tó (Lake Oxbow) is located at km 555 of river, next to the village of Timar, while the Marótzugi-Holt-Tisza (holt = dead) is located at km 568, close to the village of Gávavencsello on the left bank of the river (Fig. 1; Table 1.). The MT is naturally cut off, while the MHT was cut off during the canalization of the River Tisza in 1860. They also differ in terms of water supply, since the MT only fills up during the flooding of the Tisza, while the water balance of the MHT is managed by a sluice on an artificial canal from the River Tisza.

Both oxbows are unprotected from floods, since they are situated between dykes and the river channel. Although they are Nature Conservation areas, MHT is managed as fishpond. The MHT has been yearly stocked by about 100 – 120 kg of fish, while there is no fish stocking in the MT (*personal communications of the maintainers*). The biota of both lakes is particularly rich, but only the MHT has been registered as a national nature reserve and wildlife sanctuary (Hortobágy National Park; Pálfai, 2003).

Sampling and laboratory procedures

For the active plankton component, the sampling design aimed at integrating seasonal and spatial variations. The oxbows were sampled twice a year (May 25 and October 13, 2005; Jun 21 and October 23, 2006; April 17 and October 23, 2007; Jun 06 and October 21, 2008). Macrophyte coverage was about 80-90 % on the MT, whereas it was ~50 % on the MHT (mostly at the two shallow ends of the oxbow). *Phragmites australis* and *Typha angustifolia* inhabited the shoreline of both oxbows, so *Trapa natans*, *Nuphar luteum*, *Ceratophyllum demersum* and *Myriophyllum spicatum* were the dominant plants, although some microhabitats were also populated by *Salvinia natans*, *Potamogeton amphibium*, *Hydrocharis morsus-ranae* and *Polygonum amphibium*.

Samples were obtained for active cladoceran populations and for water quality from 15 and 16 sampling sites in the MT and MHT, respectively. Sampling sites were almost equally divided between littoral and open water (Fig. 1). Water quality samples were collected with a Meyer bottle from 1m depth, and the samples of the different sites were then pooled and the resulting sample (15-16 L) was thoroughly stirred and quantitatively subsampled for water quality analyses. Active cladocerans were sampled using a self-closing tube sampler (length: 2 m, diameter: 10 cm) with a plankton net (63 µm mesh) attached. The tube sampler is efficient in the taking of integrated plankton samples in shallow lakes; it also samples efficiently in macrophyte-beds. At each sampling site, the tube sampler was lowered to the bottom, so the entire water column (0.5-15 L, depending on depth) was filtered. Plankton samples from the different locations were pooled, resulting in the integration of within-lake variations, then they were preserved using Lugol solution. Water quality parameters (pH, conductivity, cation, anion, nutrients, COD, TOC, Chlorophyll-*a*; see in Table 3) were determined in the Laboratory of the West Transdanubian Water Authority, following the Hungarian Water Quality Standard protocols.

Sediment cores were obtained in 2008 from the deepest part of the oxbows, and the top 2 cm of sediment layers was used for the subfossil cladoceran analysis. Since sedimentation rates are high in the oxbows of the Tisza (0.2-1.1 cm; Szabó *et al.*, 2012; Korponai *et al.*, 2016), and bioturbation has significant effect on the depth distribution of remains (Kearns *et al.*, 1996), it was reasonable to assume that the top 2 cm sediment layer would represent the sampling period of active Cladocera populations in the present study.

In order to obtain a representative sample of the composition of the active cladoceran population, a minimum of 300 individuals was counted using the Utermöhl protocol. The length of the first 30 individuals of each species in each sample was measured using an ocular micrometer to an accuracy of 0.01 mm. The community size distribution of cladocerans was determined on the basis of individual body length. 2 cm^3 of sediment subsamples were deflocculated in 10 % KOH. Cladocera remains were then collected by sieving the resultant deflocculated liquid through a 35 µm mesh (Frey, 1986). Only well preserved chitinous remains (headshields, carapaces, post-abdomens, post-abdominal claws, and ephippia) were considered in the determination of the density of various Cladocera species. Furthermore, fragments were counted only if unambiguous diagnostic marks were evident. The most frequent body parts of each taxon were used to estimate the abundance of individuals in terms of density (ind/cm^3 of fresh sediment). The composition of the Cladocera community was estimated on the basis of the determination of at least 300 individuals in each subsample (Korhola & Rautio, 2001). Taxonomical identification was carried out according to work of Frey (1950), Goulden & Frey (1963), Gulyás & Forró (1999), Szeroczyńska & Sarmaja-Korjonen (2007).

Statistical analyses

To compare the water quality and median cladoceran body size of the oxbows, a non-parametric ANOVA (Kruskall-Wallis) test was conducted. Water quality components were standardized to a zero mean and unit variance. The various degrees of homogeneity of the dispersion of the active cladoceran communities of both oxbows were tested and compared using "betadisper" and "permutest" through 999 permutations. Variance partitioning in the cladoceran community was tested using the 'adonis' function. "Adonis" carries out a PERMANOVA (Permutational Multivariate Analysis of Variance Using Distance), and partitions sums of squares of a multivariate data set among sources of variation using distance matrices. In analyses the Bray-Curtis distance metric was applied to non-transformed, non-standardized cladoceran species abundance data of active communities. Following 'adonis', similarity percentages ('simper') were used to identify the species contributing most to group dissimilarity.

Species, functional and phylogenetic α-diversity

For comparison of active and subfossil communities, we calculated the mean abundances of contemporary cladoceran species of oxbows for active communities, and these data were introduced to diversity analyses. Species diversity of the active and subfossil cladoceran assemblages was estimated using Hill's numbers (Hill, 1973) on abundances, in which N0 represents species-richness (number of taxa), N1 the Shannon-Wiener index and N2 the effective number of species, which is the reciprocal of Simpson's diversity index (Hill, 1973; Jost, 2006; Telford & Birks, 2011). Values were also calculated for Pielou's evenness and the Simpson index. The Shannon-Wiener index is sensitive to dominant taxa, while the Simpson index is sensitive to rare species (Jost, 2007).

For trait diversity, the available zooplankton functional traits for all species occurring in the communities were used (Barnett *et al.*, 2007; Pomerleau *et al.*, 2015; Nevalainen & Luoto, 2017; Rizo *et al.*, 2017; Gianuca *et al.*, 2018). Combination of Rizo's and Nevalainen's traits (Nevalainen & Luoto, 2017; Rizo *et al.*, 2017) was employed in the analysis, complemented by the morphological traits of postabdominal claws (Table 2). Cladocerans use the postabdomen in the rejection of particles, the cleaning of thoracic limbs, the prevention of clogging of the filter apparatus, and in locomotion. Benthic species crawl on substrata, and use their postabdomen for pushing (Smirnov, 2017). Trait distance matrices

Table 2. Summary of functional traits used (Rizo *et al.*, 2017, Nevalainen & Luoto, 2017). *Resumen de los rasgos funcionales utilizados.*

Trait	Type of trait	Ecological function
Feeding type (S-type, D-type, C-type, I-type, B-type)	Morphological/behavioral	Feeding
Trophic regime (herbivore, detritivore, predator)	Physiological	Feeding
Size of postabdominal claws (small, intermediate, large)	Morphological	Feeding
Type of combs in postabdominal claws (no combs, hairy, fine combs, small teeth, mediate teeth, large teeth)	Morphological	Feeding
Preferred trophic status (oligotrophic, oligo-mesotrophic, mesotrophic, meso-eutrophic, eutrophic)	Physiological	Feeding
Swimming antennae length to body size ratio	Morphological	Survival
Eye size to body size ratio	Morphological	Survival
Presence of ocellus (with or without)	Morphological	Survival
Presence of color in the carapace (with or without)	Morphological	Survival
Development of defensive structure (with or without)	Morphological	Survival
Predatory escape response (pausing and jumping, rapid swimming, not moving)	Behavioral	Survival
Preferred habitat (pelagic, littoral, margins of macrophytes)	Behavioral/Feeding	Survival
Shape (globular, oval, elongated)	Morphological	Survival
Average egg clutch	Morphological	Reproduction
Body size (small <500 µm, intermediate 500–1000 µm, large >1000 µm	Morphological/physiological	Growth

using Gower's distance were calculated, because body size is a continuous variable, whereas the other traits are categorical (Borcard *et al.*, 2011). Functional trait diversities (Mason *et al.*, 2005) and Faith's phylogenetic diversities (Faith, 1992) were calculated using the functions "dbFD" and "pd" in packages "FD" (Laliberté & Legendre, 2010; Laliberté *et al.*, 2014). Faith's phylogenetic diversity is highly dependent on species richness, and so distance based phylogenetic diversity measures was chosen for use in the present study: standard effect size of mean pairwise taxon distances (sesMPD), and standard effect size of mean nearest taxon distance (sesMNTD) (Webb *et al.*, 2002), both of which provide a measure of phylogenetic diversity that is independent of species richness. The abundance weighted measures sesMPD and sesMNTD represent the average distance between two random individuals in a sample and the average distance to the closest heterospecific individual for all individuals in the sample, respectively (Webb *et al.*, 2008). The branch lengths on the phylogenetic tree were used as a measure of phylogenetic distance, while Gower dissimilarity was applied to obtain functional distances (Borcard *et al.*, 2011).

Hierarchical cluster analysis was applied to the trait matrices of cladoceran communities in order to determine potential functional groups. In the analysis, the Gowdis dissimilarity function in "FD" packages (Podani, 1999; Laliberté *et al.*, 2014) was used to compute the distance for mixed variables, and then the Ward agglomerative method was applied to attain clustering. Phylogenetic trees were created based on detailed taxonomical categories (Błędzki & Rybak, 2016) in which Sorensen distance was used in hierarchical clustering with the complete linkage agglomerative method.

Species, phylogenetic and trait ß-diversity

Sorensen and Bray– Curtis dissimilarity coefficients were calculated for the cladoceran communities of the oxbows and used as a measure of ß-diversity. Sorensen dissimilarity is based on species' presence or absence, while Bray-Curtis dissimilarity is based on the abundance of species. Next, the ß- diversity was partioned into its two components, degree of nestedness and replacement, using the bray.part function of the "betapart" statistical package (Baselga & Orme, 2012). Phylogenetic and functional ß-diversity were measured using mean pairwise taxon distances (MPD) and mean nearest taxon distance (MNTD) (Webb *et al.*, 2002). The branch lengths on the phylogenetic tree were used as a measure of phylogenetic distance, while Gower dissimilarity was applied to obtain functional distances (Borcard *et al.*, 2011). COMDIST was used for measuring phylogenetic dispersion, and COMDIST was also used to calculate functional ß-diversity metrics based on multi-trait distance

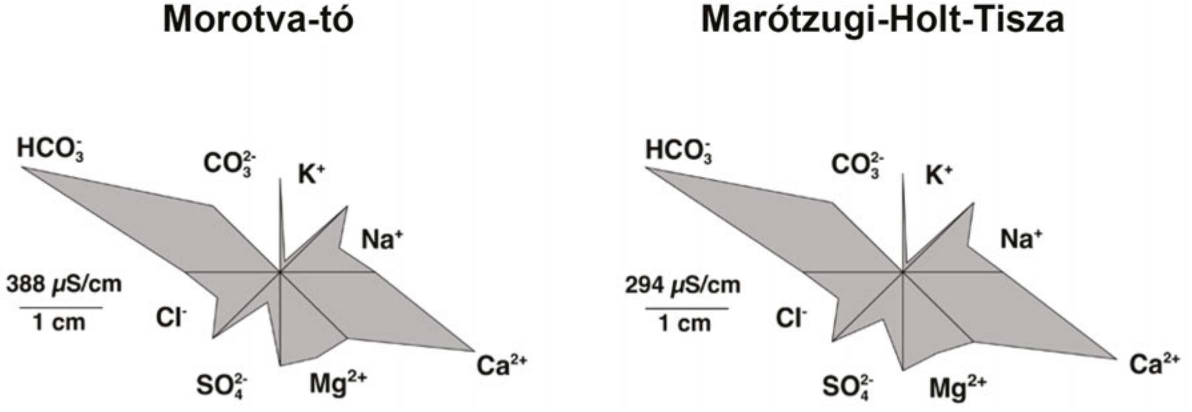

Figure 2. Maucha diagram showing the main ion composition of the two oxbows. Scale bar indicates 0.1 mm for all photographs. *Diagrama Maucha mostrando la composición iónica principal en los dos galachos.*

matrices. The abundance values for species were used when calculating both functional and phylogenetic ß-diversity. The use of COMDIST for both trait- and phylogenetically-based information allowed the use of comparable metrics for trait and phylogenetic distances (Swenson, 2014). The measures were calculated using the R package "picante" (Kembel *et al.*, 2010) in R (R Core Team, 2017).

In spite of the apparent bias in subfossil communities it is important to investigate the relationship between the distinct diversity metrics (the number of species not included) of active and sub fossil communities. Therefore, a linear model was employed to analyze the predictive strength of the diversity metrics of the subfossil community in relation to the active. Log-transformed diversity indices (log(x+1)) were entered into the model. The next step was to observe the effect of cladoceran communities (active and subfossil) or determine whether oxbows have a significant effect on the variance of diversity metrics. A linear mixed model was therefore used with type of diversity matrices as a random factor.

The degree to which the measured traits carry a phylogenetic signal was assessed using the procedures employed by Gianuca *et al.* (2018). First, a standard Mantel test is performed to assess the correlation between the phylogenetic distance and the functional trait distance matrices, then (if this test is significant ($p < 0.05$), the second step is to test whether such a correlation between phylogeny and traits is higher than that which might be expected by chance alone, given a specific evolutionary model (for more details, please see Debastiani & Duarte 2017). All calculations, data analyses and visualizations were performed in R (R Core Team, 2017), using the "betapart", (Baselga & Orme, 2012), "FD" (Laliberté & Legendre, 2010; Laliberté *et al.*, 2014)., "picante" (Kembel *et al.*, 2010), and "vegan" packages (Oksanen *et al.*, 2017).

RESULTS

Water quality

The water quality of the various oxbows was very similar in terms of common major ion composition (Fig. 2). The pH varied from neutral to slightly alkaline, but conductivity was high, and significantly higher in the MT due to a higher bicarbonate concentration. Total dissolved solids (TDS) were also significantly higher in the MT, which was due to the higher concentration of Na, K, Ca, Cl and HCO_3 ions. Although higher concentrations of TOC (total organic carbon) were found in the MT (Table 3), COD_{Cr} (chemical oxygen demand) varied over the same range in both oxbows.

Nutrient concentrations were also similar in both oxbows; they were mesotrophic, TP varied between 0.01 and 0.07 mg/l in MT, and between 0.02 and 0.06 mg/l in MHT, while chlorophyll-*a* content varied between 3 and 8 and 3 and 14 µg/l, respectively (Table 3).

Cladoceran communities

Altogether 5354 specimens were counted for active, and 1721 remains (707 specimens) were counted for subfossil community, from which 41 cladoceran species were identified. From them 23 species were common in the oxbows, while nine species were specific to each of the oxbows separately. The active cladoceran community contained almost the same species pool, while the subfossil assemblages were richer in the MT (Table 4).

Active cladoceran communities

A total of 32 cladoceran species were found in the samples, 27 in the MT and 28 in the MHT (Table 4). High degrees of variance were detected in abundance data, caused by the different times at which some species bloom (MT, 2005: *Bosmina longirostris* and *Ceriodaphnia pulchella*); MHT, 2005 and 2008: *B. longirostris*). *B. longirostris* and *Chydorus sphaericus* were found in great abundance in the oxbows. The following species were also abundant: *C. pulchella*, *Diaphanosoma brachyurum*, *Coronatella rectangula*, *Daphnia parvula* and *Pleuroxus aduncus* (Fig. 3). Three of these - *C. pulchella*, *Coronatella rectangula* and *P. aduncus* - occurred in the highest numbers in the MT, while *D. brachyurum* and *D. parvula* were found in the MHT (Fig. 3). The occurrence of twenty-one species was shared between the two oxbows (Table 4). Variance of the abundance of active communities of oxbows was homogenous (betadisper: F = 1.2801, p = 0.269), therefore the differences in the communities between the oxbows were tested for. The result revealed that the dissimilarities in active cladoceran communities were significant between the two oxbows

Table 3. Water quality parameters of the oxbows, and results of Kruskall-Wallis test for differences between the oxbows. *Parámetros de calidad del agua de los galachos y resultados del test Kruskall-Wallis entre galachos.*

	MT			**Marótzugi-Holt-Tisza**			**Kruskall-Wallis**		
	min	**mean**	**max**	**min**	**mean**	**max**	χ^2	**df**	***p***
HCO3 (mg/l)	176	215.63	278	126	152	168	11.294	1	**0.001**
TDS (mg/l)	185	257.25	326	140	185.88	262	6.362	1	**0.012**
cond (µS/cm)	293	387.75	477	244	293.75	337	6.1	1	**0.014**
Ca (mg/l)	39	51.25	59	35	41.75	52	5.625	1	**0.018**
Cl (mg/l)	16	30.38	41	12	22.13	27	5.121	1	**0.024**
TOC (mg/l)	5.6	7.09	9	4.8	5.82	7.1	4.412	1	**0.036**
K (mg/l)	3.2	5.74	15.6	2.4	3.54	4.9	4.219	1	**0.04**
SS (mg/l)	0	2.61	10	0.1	9.48	29	4.231	1	**0.04**
Na (mg/l)	12	17.875	25	9	14.5	18	3.052	1	0.081
Mg (mg/l)	2.2	13.9	26	8	9.75	14	2.53	1	0.112
TDP (mg/l)	0.006	0.018	0.038	0.006	0.026	0.058	2.184	1	0.139
Chl-a (µg/l)	3	5.63	8	3	9.25	14	2.04	1	0.153
TN (mg/l)	0.57	0.82	1.05	0.46	0.73	1.04	1.337	1	0.248
DIN (mg/l)	0.04	0.075	0.107	0.056	0.096	0.173	0.799	1	0.371
COD$_{Cr}$ (mg/l)	7.5	19.88	36	7.5	17.63	29	0.344	1	0.557
Mn (mg/l)	0	0.03	0.18	0.005	0.016	0.07	0.147	1	0.702
TP (mg/l)	0.006	0.038	0.071	0.02	0.037	0.064	0.025	1	0.875
pH	7.1	7.56	8	7.1	7.55	8.1	0.012	1	0.914
DIP (mg/l)	0.002	0.011	0.032	0.002	0.013	0.047	0.012	1	0.914
SO4 (mg/l)	12.5	20.12	54	12.5	22.25	59	0.003	1	0.955
Fe (mg/l)	0	0.05	0.17	0.005	0.03	0.06	0	1	1
Secchi depth (cm)	56	92	125	28	62	95	42.216	1	**<0.001**

Table 4. List of species occurring in source and sediment samples in the oxbows. (+: present; -: absent; e: ephippia only). *Lista de especies presentes en las muestras de origen y de sedimento en los galachos.*

	Morotva-tó		**Marótzugi-Holt-Tisza**	
	source	**sediment**	**source**	**sediment**
dominant species				
Bosmina longirostris (O. F. Müller, 1776)	+	+	+	+
Chydorus sphaericus (O. F. Müller, 1776)	+	+	+	+
species in Marótzugi-Holt-Tisza				
Moina micrura Kurz, 1875	-	-	+	-
Daphnia ambigua Scourfield 1947	-	-	+	-
Disparalona rostrata (Koch, 1841)	-	-	+	-
Ceriodaphnia laticaudata P. E. Müller, 1867	-	-	+	e
Alonella excisa Fischer, 1854	-	-	+	-
Camptocercus rectirostris (Schödler, 1862)	-	-	-	+
Leydigia leydigi (Schödler, 1863)	-	-	-	+
Ceriodaphnia dubia Richard, 1894	-	-	+	-
Alona costata Sars, 1862	-	-	-	+
species in MT				
Simocephalus serrulatus (Koch, 1841)	+	-	-	-
Alonella nana (Baird, 1843)	-	+	-	-
Monospilus dispar Sars, 1862	-	+	-	-
Scapholeberis mucronata (O. F. Müller, 1776)	+	-	-	-
Eurycercus lamellatus (O. F. Müller, 1776)	+	-	-	-
Pseudochydorus globosus (Baird, 1843)	+	-	-	-
Alona quadrangularis (O. F. Müller, 1776)	-	+	-	-
Leydigia acanthocercoides (Fischer, 1854)	-	+	-	-
Oxyurella tenuicaudis (Sars, 1862)	-	+	-	-
common species				
Ceriodaphnia pulchella Sars, 1862	+	-	+	-
Coronatella rectangula Sars, 1862	+	+	+	+
Diaphanosoma brachyurum (Liévin, 1848*)*	+	-	+	-
Daphnia parvula Fordyce, 1901	+	-	+	-
Pleuroxus aduncus (Jurine, 1820)	+	-	+	+
Acropterus harpae (Baird, 1834)	+	+	+	+
Simocephalus vetulus (O. F. Müller, 1776)	+	-	+	-

Cont.

Table 4. (cont.)

Alonella exigua (Lilljeborg, 1853)	+	+	+	-
Ceriodaphnia quadrangula (O. F. Müller, 1785)	+	-	+	-
Daphnia galeata Sars, 1863	+	-	+	-
Pleuroxus truncatus (O. F. Müller, 1785)	+	+	+	-
Pleuroxus trigonellus (O. F. Müller, 1776)	+	+	+	-
Pleuroxus laevis Sars, 1862	+	+	+	+
Alona affinis (Leydig, 1860)	+	+	+	+
Alona guttata Sars, 1862	+	+	-	+
Graptoleberis testudinaria (Fischer, 1848)	+	+	+	+
Simocephalus expinosus (De Geer, 1778)	+	-	+	-
Ceriodaphnia reticulata (Jurine, 1820)	+	+	+	-
Bunops serricaudata (Daday, 1884)	+	-	+	-
Alona intermedia Sars, 1862	+	+	+	-
Iliocryptus sordidus (Liévin, 1848)	+	-	+	-

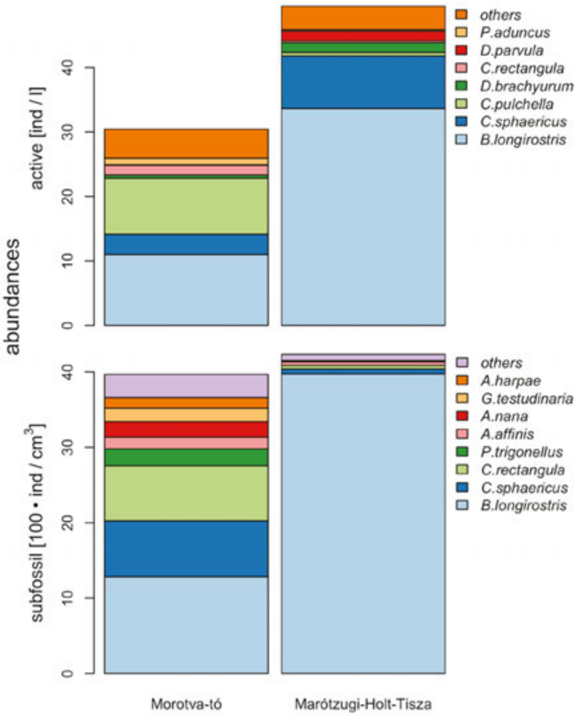

Figure 3. Cumulative abundances of dominant cladoceran species in the oxbows. *Abundancia acumulada de las especies dominantes de cladóceros en los galachos.*

(Permanova: $F_{model} = 2.93$, $R^2 = 0.173$, $p = 0.007$). The SIMPER analysis identified a decreasing order, in which *B. longirostris*, *C. pulchella* and *C. sphaericus* contributed most to the dissimilarity between oxbows. The high abundance of *B. longirostris* and *C. spharicus* in MHT was responsible for dissimilarities of 50 % and 10 %, respectively, while the high abundance *C. pulchella* contributed 17 % of the total dissimilarity of the MT. Due to the dominance of small bodied cladocerans, average cladoceran body size was small in both oxbows (MT: 0.443 mm (n = 892), MHT: 0.383 mm (n = 814)), but it was significantly smaller in the MHT (Kruskal-Wallis test: $\chi^2 = 12.346$, df = 1, $p < 0.001$). Although the average body sizes of both dominant species (*Bosmina longirostris*, and *Chydorus sphaericus*) were significantly smaller in the MHT (*Bosmina longirostris*: MHT: 0.268 mm, MT: 0.290 mm, Kruskal-Wallis test: $\chi^2 = 15.162$, df = 1, $p < 0.001$; *Chydorus sphaericus*: MHT: 0.278 mm, MT: 0.293 mm, Kruskal-Wallis test: $\chi^2 = 5.3082$, df = 1, $p = 0.02$), the proportion of large cladocerans (1 mm = <) played a significant role in the body size spectra of cladocerans in the MT (Fig. 4).

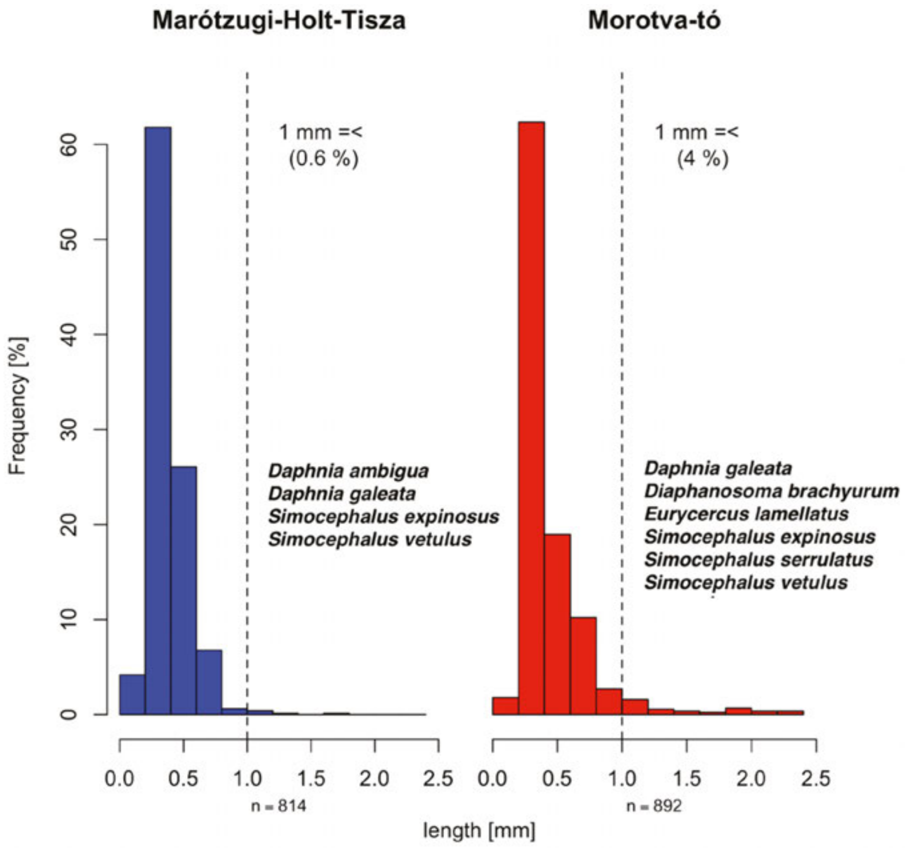

Figure 4. Body size distribution of cladoceran communities. *Distribución de tamaño de la comunidad de cladóceros.*

Subfossil cladocerans

Remains of a total of 23 cladoceran species were found in the sediment samples, of which 18 species occurred in the MT, and 13 in the MHT (Table 4.). Most of the remains belonged to the dominant species *Bosmina longirostris*, *Chydorus spharicus*, and *Coronatella rectangula* in both oxbows. More remains of *B. longirostris* were found in the MHT, whereas those of *C. rectangula* and *Chydorus spaericus* were most numerous in the MT.

As found in the active cladoceran communities, differences were also found in the subfossil communities of the two oxbows. The remains of *Alonella nana*, *Monospilus dispar*, *Alona quadrangularis*, *Leydigia acanthocercoides* and *Oxyurella tenuicaudis* were found in the MT exclusively, while the remains of *Leydigia leydigi*, *Alona costata* and *Ceriodaphnia ephippia* only occurred in the MHT (Table 4). A simper analysis revealed that *B. longirostris*, and *C. rectangula* and *C. sphaericus* contributed to the dissimilarity of the cladoceran communities in the oxbows. A greater abundance of *B. longirostris* in the MHT accounted for the greatest proportion (51 %) of the total dissimilarity between the oxbows, while the remains of *C. rectangula* and *C. sphaericus* were found in a higher numbers in the MT, and thus they contributed 13 % and 12 % to the total dissimilarity, respectively.

Diversity

Homogenous dispersion of contemporary data allowed merging the active and subfossil species sets, altogether 32 species composed the pooled community of the oxbows. The active community

Table 5. Diversity indices of cladoceran communities in the oxbows. (higher values are in bold.). *Índices de diversidad de las comunidades de cladóceros en los galachos(los valores más altos están en negrita).*

	Morotva-tó		**Marótzugi-Holt-Tisza**	
	source	**sediment**	**source**	**sediment**
nbsp	27.000	18.000	28.000	13.000
sing.sp	27.000	18.000	28.000	13.000
FRic	0.979	0.927	**0.981**	0.770
qual.FRic	0.580	0.726	0.580	0.726
FEve	**0.542**	0.547	0.498	**0.689**
FDiv	**0.949**	0.834	0.877	**0.985**
FDis	**0.278**	**0.246**	0.193	0.063
RaoQ	**0.083**	**0.069**	0.059	0.018
Shannon	**1.958**	**2.051**	1.254	0.363
J	**0.073**	**0.114**	0.045	0.028
Simpson	**0.771**	**0.815**	0.509	0.118
N2	**4.366**	**5.403**	2.035	1.134
PD	**3.176**	**1.755**	3.056	1.528
sesMPD.P	**0.452**	**0.357**	0.325	0.068
sesMNTD.P	0.252	0.280	**0.445**	**0.551**
sesMPD.T	**0.346**	**0.294**	0.237	0.064
sesMNTD.T	0.166	0.186	**0.255**	**0.372**

consisted of 27 and 28 species in the MT and MHT, respectively. The larger part of the active communities (MT: 50 % and MHT: 60 %) did not leave any remains in the sediment. In contrast to active communities, only five (MT) and four (MHT) species were exclusively found in subfossil remains. Consequently, 13 species were recorded from remains and from plankton samples in the MT, while nine species were found in plankton and sediment samples in the MHT. Although the species composition of the active cladoceran communities was similar, ~20 % species are not shared between the two oxbows, the species diversity components of active communities were higher in the MT (Table 5). Subfossil cladocerans were represented by fewer species, and the communities of the two oxbows were noticeably unalike. Fewer species were identified in the subfossil community in the MHT, moreover the proportion of not-shared species exceeded the number of local species. All species diversity indices were higher in the MT (Table 5). The functional diversity of the active community was quite similar in both oxbows. Functional richness was similar, but the other components of functional diversity, except functional divergence (FDiv), were higher in the MHT (Table 5). All functional diversity indices of the subfossil community, except functional evenness (FEve) and functional divergence (FDiv) were also higher in the MT (Table 5). Faith's phylogenetic diversity was higher in both active and subfossil communities in the MT (Table 5).

All distance-based phylogenetic diversity metrics indicated a higher degree of diversity of the active community of the MT. This not was true of the subfossil community of the MHT, where a higher sesMNTD was found. Significantly fewer species were found here, thus absent species tended to decrease the sesMPD, but increase the sesMNTD, increasing the distance between the closest relatives in the phylogenetic tree. This was observed when sesMPD and sesMNTD were used for trait diversity (Table 5).

A linear model revealed that the subfossil communities correctly predicted the diversity metrics of the active community. A significant relationship was found in both oxbows, but it was stronger in the case of the MT (MHT: $R^2 = 0.709$, $F_{1,13} = 35$, $p < 0.001$; MT: $R^2 = 0.923$, $F_{1,13} = 168$, p- < 0.001). The linear mixed model revealed that both oxbows had a significant effect on diversity indices (community: $F_{1,42} = 2.41$, $p = 0.128$; oxbow: $F_{1,42} = 7.88$, $p = 0.008$; type:oxbow: $F_{1,42} = 1.48$, $p = 0.229$). The cladoceran communities of the two oxbows presented a high degree of similarity ($ß_{SOR.A} = 0.2$ and $ß_{SOR.S} = 0.4839$ for active (A) and subfossil communities (S), respectively), but abundances significantly increased ß-diversity ($ß_{BC.A} = 0.572$; $ß_{BC.S} = 0.6379$). A high portion of common species was found that was expressed in low turnover and low nestedness components

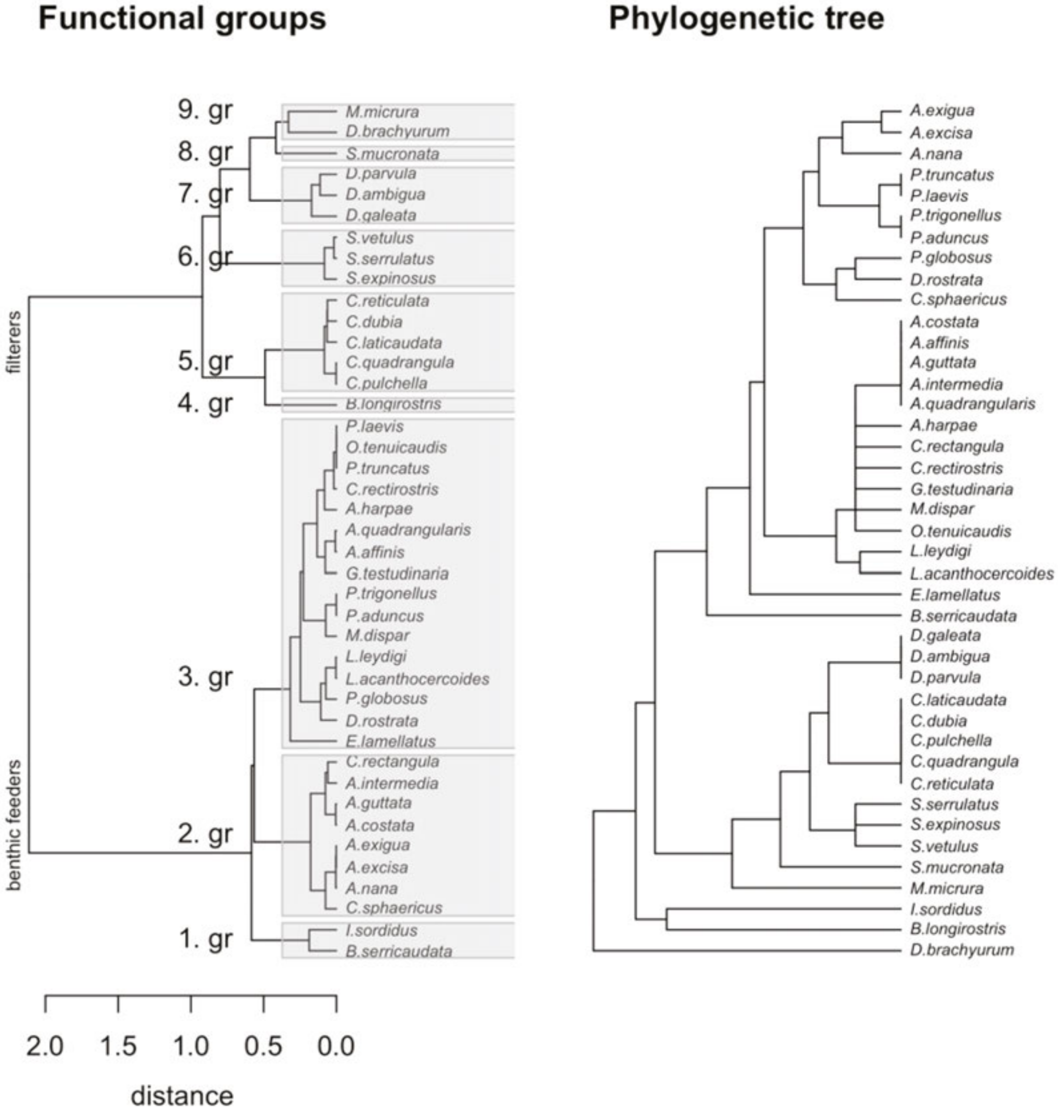

Figure 5. Functional dendrogram (left panel) and phylogenetic tree (right panel) of compound cladocera communities. Taxonomy based on Błędzki & Rybak (2016). Branch lengths are representative only, and do not represent quantitative values. *Dendrograma funcional (panel izquierdo) y árbol filogenético (panel derecho) de la composición de la comunidad de cladóceros.*

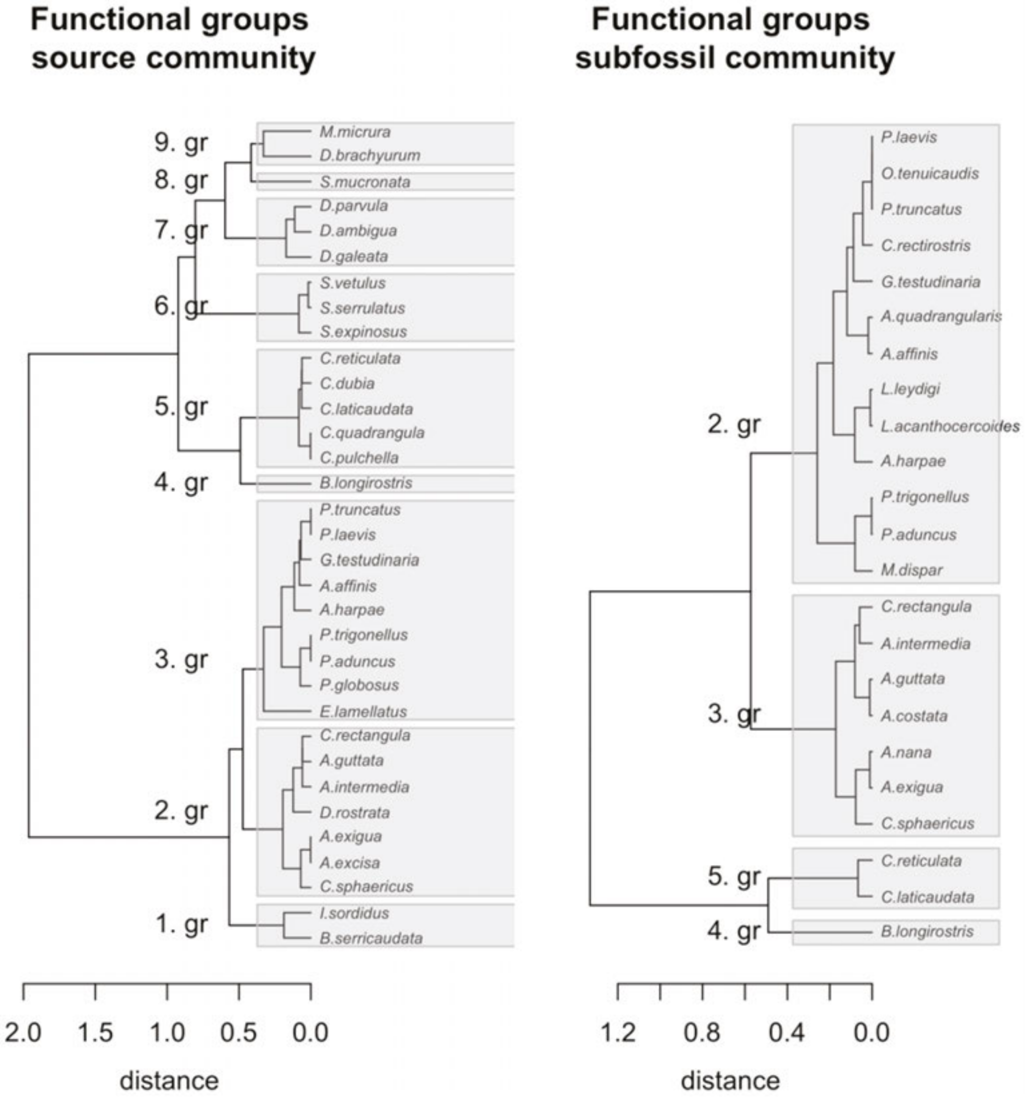

Figure 6. Functional dendrogram of active (left panel) and subfossil (right panel) communities. *Dendrograma funcional de las comunidades activas (panel izquierdo) y subfósiles (panel derecho) de cladóceros.*

(turnover: ß$_{SIM.A}$ = 0.1852, ß$_{SIM.S}$ = 0.3846; nestedness: ß$_{SNE.A}$ = 0.0148, ß$_{SNE.S}$ = 0.0993) of ß-diversity. The abundance component of ß-diversity revealed the importance of abundant species, since the balanced variation components of ß-diversity have high values (ß$_{BC.BAL.A}$ = 0.4382, ß$_{BC.BAL.S}$ = 0.6258). The abundance-gradient component of ß-diversity of the subfossil communities (ß$_{BC.GRA.S}$ = 0.012) was lower than that of the active community (ß$_{BC.GRA.A}$ = 0.134), indicating a low degree of nestedness in the subfossil cladoceran community. The oxbows presented higher degrees of dissimilarity in the phylogenetic structures of active communities (COMDIST$_{active}$ = 0.515) than the subfossil one (COMDIST$_{subfossil}$ = 0.436).

Functional groups

Nine functional groups were established for cladocerans (Fig. 5). The two main branches correspond to the feeding modes of those species. Filterers (herbivorous species) were further subdivided into six groups: small planktonic bacteria feeders (Gr. 4, *Bosmina*); small littoral daphnid filterers (Gr. 5, *Ceridaphnia*); littoral large filterers (Gr. 6, *Simocephalus*); pelagic large filterers (Gr. 7, *Daphnia*); littoral hyponeuston scrapers (Gr. 8, *Scapholeberis*) and pelagic non-daphnid filterers (Gr. 9, *Diaphanosoma* and Moinidae). The benthic feeder (detritivorous-herbivorous) group is comprised of a cluster of benthic pickers (Gr. 1, Ilyocryptidae and Macrothricidae) and substratum scrapers (Chydoridae). The latter group may be further divided into two groups in terms of body length: small substratum scrapers (Gr. 2, small Chydorinae and small Aloninae), and large substratum scrapers (Gr. 3, large Aloninae and large Chydorinae; (Fig. 5).

All nine functional groups were represented in the active communities, while the subfossil communities shared only four groups (Fig. 6). In the MT, small planktonic bacteria feeders accounted for the highest portion of the cladoceran community (44 %); the next group, small littoral filterers, for 27 %, large benthic scrapers for 20 %, small benthic scrapers for 5 %, large littoral filterers for 2.5 %, and large planktonic filterers for only 1 % of the cladoceran community. In the MHT, small planktonic bacteria feeders made up the major proportion (77 %) of the active cladoceran community. Large benthic scrapers (17 %), small littoral filterers and small benthic scrapers accounted for 15 % each, and small planktonic filterers made up 2 % of the source community (Fig. 7). The abundance of the subfossil community was not equally shared between the three functional groups. The prevalence of small planktonic bacteria feeders was overwhelming, at 94 %, while large and small benthic scrapers made up the remainder in equal proportions (Fig. 7).

A standard Mantel test revealed strong correlation between trait and phylogenetic distances (active: mantel r = 0.715, $p < 0.001$; subfossil: mantel r = 0.845, $p < 0.001$). In addition, an EM-Mantel test indicated that the following traits are more conserved along the phylogeny than might be expected in a Brownian motion evolutionary model ($p < 0.05$): swimming antennae length, predatory escape response, ocellus presence, colored carapace, feeding type and trophic regime.

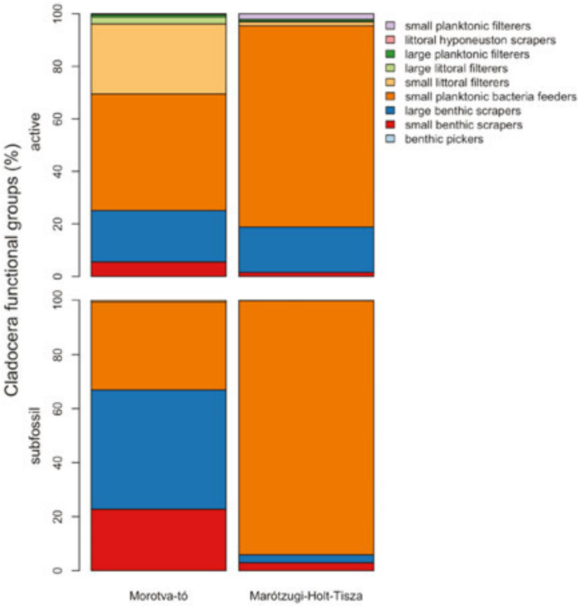

Figure 7. Prevalence of cladoceran functional groups in the oxbows. *Predominio de los grupos funcionales de cladóceros en los galachos.*

DISCUSSION

Both the Morotva-tó (MT) and Marotzugi-Holt-Tisza (MHT) are situated on the floodplain of the River Tisza. They can be filled either indirectly by groundwater, or directly by sluice from the main channel of the river (Babka *et al.*, 2011). Correspondingly, the water ion composition of the two oxbows is almost identical due to the effect of the River Tisza (Fig. 2; Table 2). Babka & Szabó (2007) found that the nutrients of unprotected oxbows were within the range of those to be found in the River Tisza. The nutrient contents were low (Table 3) in both investigated oxbows; consequently low chlorophyll-*a* content was measured, and so the aquatic ecosystems of the oxbows may be said to be under mesotrophic conditions. Although the chlorophyll-*a* concentration in the oxbows did not differ significantly, its average content was higher and varied over a wider range in the MHT; moreover, lower transparency was measured in this oxbow (Table 3). This phenomenon can be explained by the different trophic structure of the two oxbow ecosystems.

Differences in cladoceran communities in the waterbodies can be explained by nutrients (algae) and predators (invertebrate or/and fish). Invertebrate predators significantly increase the size of cladocerans, while fish decrease it. The mean cladoceran body size was smaller, and large cladocerans (1 mm = <) occupied a negligible part (less than 1 %) of the body size spectrum in the MHT (Fig. 6), demonstrating the effects of fish predation pressure on the cladoceran community. Besides the effect on the size distribution, fish exhibit a strong effect on the structure of the species composition of cladoceran community, as well (Brooks & Dodson, 1965; Dodson, 1974). Small sized cladoceran species (*Bosmina*, small daphnids and *Chydorus sphaericus*) will spread and become dominant in the cladoceran community under high fish predation pressure (Christoffersen *et al.*, 1993). In the studied oxbows *Bosmina longirostris* and *Chydorus spaericus* were the dominant species. These species are very common and abundant in shallow waters and in the littoral zone, and could not be considered as an indicator of the dimensions of the pelagic zone (Hofmann, 1983, Vijverberg & Boersma, 1997, Błędzki & Rybak, 2016). Populations of these species were two (*C. sphaericus*) or three (*B. longirostris*) times higher in the MHT than in the MT, while their median body size was smaller in the MHT.

High macrophyte coverage could also be considered evidence of low fish stocks. Many papers have revealed that macrophyte expansion follows fish reduction in shallow lakes (Jeppesen *et al.*, 1997; Scheffer, 2001; Lauridsen *et al.*, 2003; Davidson *et al.*, 2010; Sayer *et al.*, 2010). Moreover, biomanipulation experiments have also revealed that a reduction in planktivorous fish populations increases water transparency, since dense zooplankton filters out phytoplankton, causing a clear water phase (Lauridsen *et al.*, 2003; Jeppesen *et al.*, 2007, 2012; Nykänen *et al.*, 2010). Secchi depths in the MT were significantly higher (Table 3), also suggesting a lower planktivorous fish stock. Macrophytes can stabilize the clear water phase and extended macrophyte beds are preferred habitats for phytophylous cladoceran species such as *Simocephalus expinosus*, *S. serrulatus* or *Eurycercus lamellatus*, which occurred exclusively in the MT. Other phytophylous cladocerans such as *Acroperus harpae*, *Alona affinis*, *A. guttata*, *Graptoleberis testudinaria*, and *Pleuroxus species* (except *P. truncatus*) indicated macrophyte habitats in both oxbows, but their greater abundance corresponded to the expanded (80-90 % coverage) macrophyte beds in the MT. Such thermophylous planktonic cladocerans as *Diaphanosoma brachiurum* occurred in greater numbers in the MHT, while *Moina micrura* was found in the MHT only; therefore, their presence was related to open water (macrophyte coverage ~50 %) in this oxbow. The occurrence of ceriodaphnids in both oxbows also indicates macrophyte belts; however, higher abundances of ceriodaphnids indicate higher macrophyte cover in the MT (Fig. 3). Consequently, the results of the investigation of body size distribution, macrophyte coverage and species composition of cladocerans indicate differences in fish predation pressure between the two oxbows (Christoffersen *et al.*, 1993, Vijverberg & Boersma, 1997; Nevalainen & Luoto, 2017). It was therefore concluded that the fish stock was probably higher in the MHT oxbow.

The functional structure of cladoceran communities may be a better predictor of ecosystem functioning than the taxonomical (Schleuter *et al.*, 2010; Nevalainen & Luoto, 2017; Nevalainen *et al.*, 2018). Nine functional groups were distinguished in the species pool of the oxbows (Fig. 4). This structure presents a strong evolutionary background. A majority of the traits is phylogenetic in origin. For example, the structures of thoracic limbs are morphological traits in the systematics of cladocerans (DeMott & Kerfoot, 1982; Barnett *et al.*, 2007; Dumont, 2016). Moreover, these are a part of the filtering apparatus, and therefore may be considered a feeding trait (Rizo *et al.*, 2017). The transparency of the cladoceran body, and its helmets, swimming speed and size of eye are all connected with the cladocerans' defensive strategy against predators (Zaret & Kerfoot, 1975; Spaak, 1997; Boersma *et* *al.*, 1998; Rhode *et al.*, 2001; Kappes & Sinsch, 2002; Kerfoot & Weider, 2004; Laforsch & Tollrian, 2004; Sakamoto & Hanazato, 2008; Litchman *et al.*, 2013).

Chydorids and bosminids comprised the greater part of cladocerans in terms of abundance and the traits shared by the most abundant species. *Bosmina longirostris* was the most abundant planktonic species in active and subfossil communities, and its abundance was higher in the MHT, therefore its evolutionary conserved traits (transparency, specific feeding type, no-ocellus, small body) became the dominant traits in this oxbow (Gianuca *et al.*, 2018). The functional trait diversity indices of the active and subfossil communities were generally high in both oxbows, but they were a little higher in the MT. Functional diversity indices revealed that the cladoceran community in the MT has a more complex trait structure. The degree of functional divergence indicates the presence to some extent of niche differentiation, and thus resource competition. Communities with high functional divergence may therefore have increased levels of ecosystem function as a result of more efficient resource use (Mason *et al.*, 2005). Functional divergence is therefore sensitive to extremities in abundance. The occurrence of the most abundant species leads to extremities in functional traits. *Bosmina longirostris*, *Chydorus sphaericus* and *Diaphanosoma brachyurum* were the most abundant species in the active communities of both oxbows, but they were the only species represented by high abundance (> 33, 8 and 1.5 ind/l, respectively) in both oxbows, while the abundance of these species was much lower (~10, 3 and 0.4 ind/l) in the MT. Furthermore, *Ceriodaphnia pulchella*, *Coronatella rectangular* and *Pleuroxus aduncus* also occurred in considerable numbers (their abundances varied between ~9, 1.5, and 1 ind/l respectively). The above mentioned cladocerans represent distinct trait matrices and phylogenetic background, and so they played a significant role in the degree of functional divergence in the source community in this oxbow. The degree of functional divergence of the subfossil community was also high, and even higher than the active community in the MHT, since *B. longirostris* was the only species with extremely high abundance (3975 ind/cm^3). The abundance of the rest of the cladocerans did not exceed 10 ind/cm3 in this oxbow. *B. longirostris* was the most abundant cladoceran in the MT, similarly to the source community. The functional divergence of subfossil communities in this oxbow was lower, due to seven more species occurring in high abundance (> 100 ind/cm^3) (Table 5). Functional dispersion is the weighted mean distance of individual species to the centroid of all species in a given trait space, in which the weights are species relative abundances (Laliberté and Legendre, 10). In the trait space the centroid moves toward the species with highest abundance, reducing the distance of this species from the centroid, and functional dispersion also decreases. In the MHT, *B. longirostris* had an extremely high abundance, and so it decreased the FDis of both active and subfossil communities of the oxbow. In the MT, more species had high abundances, so species with high abundance could not attract the centroid toward themselves, and the FDis of the active and subfossil community were higher (Table 5).

Faith's phylogenetic diversities of both cladoceran communities were higher in the MT, since the communities were composed of phylogenetically distinct species (Table 5). Since PD is highly dependent on species richness, and subfossil cladoceran communities had lower species richness, the low PD in oxbows corresponds to the low degree of species richness in their subfossil communities. All distance based phylogenetic diversity metrics presented higher degrees of diversity of the active community of the MT. This not was true of the subfossil community of the MHT, where a higher sesMNTD was found. Significantly fewer species were found here, thus absent species decreased the sesMPD, but increased the sesMNTD, increasing the distance between the closest relatives in the phylogenetic tree. This was observed when sesMPD and sesMNTD were used for trait diversity (Table 5).

Subfossil communities display a bias in community structure compared to active communities, mainly due to taphonomy and habitat heterogeneity (Korhola & Rautio, 2001; Kattel *et al.*, 2007; Nevalainen, 2011). Planktonic species are underrepresented in the subfossil communities, since their shells are soft and less chitinized, and therefore less able to resist decomposition processes. Although fewer species were found in the sediment samples, some species were only present as remains, and thus subfossil communities contribute 20 % of the local species pool. Species such as *Alonella nana*, *Alonella quarangularis*, *Camptocercus rectirostris*, *Leydigia*, *Monospilus dispar* and *Oxyurella tenuicaudis* are rarely found in active community samples, but their remains are very common in sediment samples.

To answer the question of how well the diversity of a subfossil community reflects that of the active one, the measured diversity indices of active and subfossil communities were compared. The high correlations could be explained by the great degree of similarity of the communities, corresponding to the low turnover component of ß-diversity. The cladoceran communities of the studied oxbows behaved differently in terms of the active-subfossil relationship. Those diversity metrics which depend on species richness occupy a position far from the 1:1 line (species α-diversity metrics such as Shannon entropy, the Simpson dominance index, Hill's effective number of species, N2, and Faith's phylogenetic diversity). Those metrics that are either distance-based or tree-based measurements of diversity are close to the 1:1 line (Fig. 8), suggesting that they are useful in paleolimnological reconstruction.

The investigated the oxbows were populated by fish exercising a strong effect on cladoceran populations, and trophic status of both lakes are

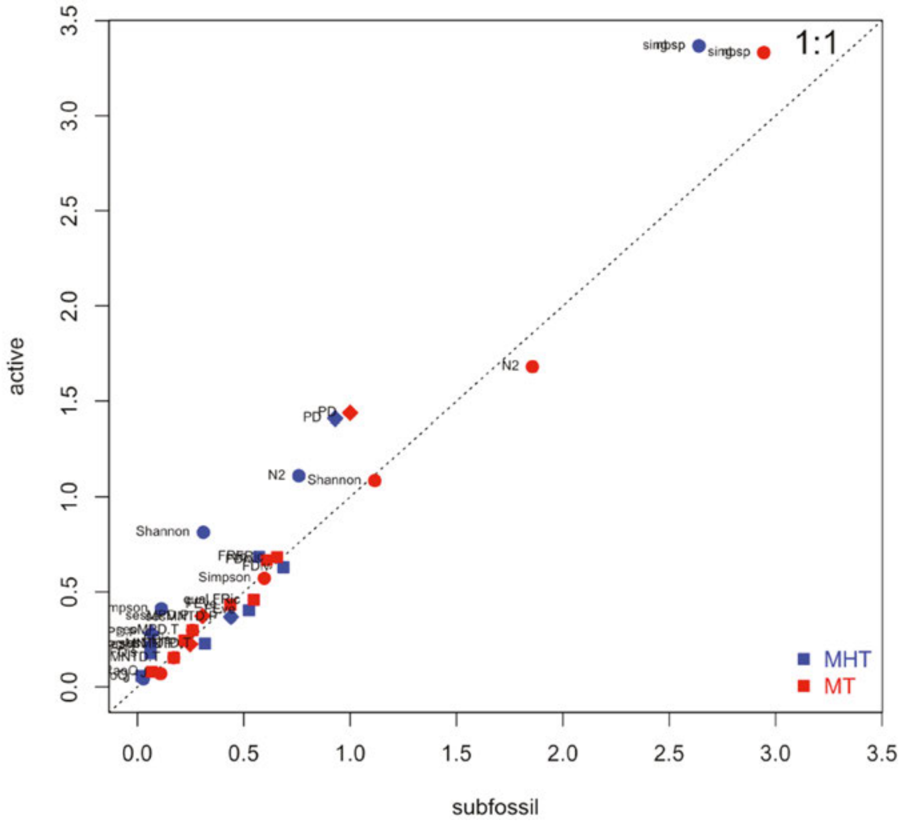

Figure 8. Scatterplot of diversity indices of active against subfossil cladoceran communities. (filled circles: species diversity indices; filled squares: functional diversity indices; diamonds: phylogenetic diversity indices). *Gráfico de dispersión de los índices de diversidad de las comunidades de cladóceros activas contra las comunidades subfósiles. (círculos: índices de diversidad de especies; cuadrados: índices de diversidad funcional; rombos: índices de diversidad filogenética).*

mesotrophic, fish (top-down force) is more likely to be an environmental filter than nutrients (bottom-up force) having the effect on cladoceran communities. They differed in terms of ecosystem functioning (strength of top-down forces). The MHT is operated as fishpond, while the MT is not; the MT is almost fully covered by macrophytes, while the MHT is much less so. Diversity metrics revealed the contrast in the ecosystem functioning of the oxbows since a higher degree of diversity was measured in the MT in the case of almost all indices used in the investigation.

CONCLUSIONS

Bias between the active population of cladoceran species (source community) and the subfossil cladoceran community were investigated. Heavy chitinized species' live remains occur in the sediment, therefore biases may be found in the reconstructed communities. Biases are caused by, on the one hand, body parts of soft bodied filterers (Daphniidae, Moinidae, Sididae (*Diaphanosoma* sp.)) being underrepresented in the subfossil remains of cladocerans, while on the other hand, species which are hard to collect are underrepresented or absent in contemporary samples. These latter are mostly found in littoral, are benthic or phytophylous species. Both circumstances can have a strong effect on diversity assessment. In spite of such biases, the sediment community may reflect ecosystem functioning. The subfossil cladoceran community in the MT corresponded to low fish densities and the high macrophyte coverage, representing weaker top-down forces in this oxbow. The phylogenetic and functional approach to subfossil cladocerans should prove useful in paleolimnological reconstruction, since phylogenetic and trait based diversity indices predicted properly the top-down regulation as an ecosystem function.

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Remote sensing images for water quality studies

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Monitoring the ecological state of a hypertrophic lake (Albufera of València, Spain) using multitemporal Sentinel-2 images

Xavier Sòria-Perpinyà[1,*], Patricia Urrego[2], Marcela Pereira-Sandoval[2], Antonio Ruiz-Verdú[2], Ramón Peña[2], Juan M. Soria[1], Jesús Delegido[2], Eduardo Vicente[1] and José Moreno[2]

[1] Image Processing Laboratory, Universitat de València. C/ Catedrático José Beltrán Martínez, 2. 46980, Paterna, València.
[2] Cavanilles Institute of Biodiversity and Evolutionary Biology (ICBiBE). Universitat de València. C/ Catedrático José Beltrán Martínez, 2. 46980, Paterna, València.

[*] Corresponding author: Javier.Soria-Perpina@uv.es

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ABSTRACT

Monitoring the ecological state of a hypertrophic lake (Albufera of València, Spain) using multitemporal Sentinel-2 images

Albufera of València, a hypertrophic lake, has been studied extensively since the 1980s, but the efforts to revert the system to a clear water state have not yielded the expected results because pressure on this system is growing (increase in nutrient-rich water inputs, decrease in precipitation and increase in evaporation). The current state of the lake requires constant monitoring, and one of the main biological parameters used in ecology and water management to monitor and control the ecological status of aquatic ecosystems is chlorophyll *a* concentration [Chl-*a*]. In this sense, remote sensing is an optimal tool for continuous monitoring of the quality state of the water body through [Chl-*a*] and to obtain a better understanding of its spatial dynamics. This work aims to demonstrate the validity of an algorithm for [Chl-*a*] retrieval from Sentinel-2 (A and B), the new Earth observation satellites of the European Space Agency, with the sensor MSI, multispectral (13 bands) from 404 nm to 2200 nm, a spatial resolution of 10 m and a temporal frequency of 5 days - values unthinkable until now as regards to open access images. The study was carried out with images from 2016 and 2017, but only 40 images out of the 81 taken by the satellite could be used – such rate was mainly due to unfavourable weather conditions. Once images were downloaded, the SNAP 5 software was used for the processing. Using the Sen2cor tool, they were corrected atmospherically and, with the algorithm developed by Soria *et al.* 2017, lake [Chl-*a*] was estimated. Estimated data were validated against field samples: a total of 18 sampling campaigns were carried out and 92 samples were taken to measure the [Chl-*a*]. In addition, to better interpret results, data on conductivity and Secchi disk depths measurements were taken in the field and hydrological, precipitation and wind data were also collected. Results of the validation were deemed very good since an R = 0.8 was obtained when applying a linear correlation between field data and estimates, which shows the robustness of the algorithm used. From the interpretation of the thematic maps, it was possible to infer that the temporal evolution in [Chl-*a*] variations follows an annual bimodal pattern, where the decrease in [Chl-*a*] is determined either by a significant increase in water renewal of the lake or by the depletion of the available nutrients in the water due to a previous excessive growth of phytoplankton.

Key words: eutrophication, water quality, water management, chlorophyll-*a*, remote sensing

RESUMEN

Seguimiento del estado ecológico de un lago hipertrófico (Albufera de València, Spain) usando imágenes multitemporales de Sentinel-2

El lago de la Albufera de València, un lago hipertrófico, ha sido largamente estudiado desde los años 80, pero los esfuerzos en revertir el sistema a un estado de aguas claras no han dado los frutos esperados debido a que la presión sobre este sistema es cada vez mayor (aumento de aguas ricas en nutrientes, disminución de la precipitación y aumento de la evaporación). El *estado en que se encuentra el lago requiere un seguimiento constante, y uno de los principales parámetros biológicos utilizados en ecología y gestión del agua para el seguimiento y control del estado ecológico de los ecosistemas acuáticos es la concentración de clorofila* a *[Chl-*a*]. En este sentido, la teledetección es una óptima herramienta para el seguimiento continuo del estado de calidad de la masa de agua a través de la [Chl-*a*], permitiendo además, entender mejor su dinámica espacial. En este trabajo se pretende demostrar la validez de un algoritmo para la estimación de [Chl-*a*] a partir de imágenes de Sentinel-2 (A y B), los nuevos satélites de observación de la tierra de la Agencia Espacial Europea, con el sensor MSI, multiespectral (13 bandas) desde 404 nm hasta 2200 nm, una resolución espacial de 10 m y una frecuencia temporal de 5 días, impensables hasta ahora en imágenes de libre acceso. El estudio se realizó con imágenes de los años 2016 y 2017. Durante este periodo solo se pudieron aprovechar 40 imágenes sin nubes de las 81 que tomó el satélite. Una vez descargadas las imágenes, se utilizó el programa SNAP 5 para su procesado; mediante la herramienta Sen2cor se corrigieron atmosféricamente y con el algoritmo elaborado por Soria* et al. *2017 se estimó la [Chl-*a*] del lago. Los datos estimados se validaron con muestras de campo: se realizaron un total de 18 muestreos y se tomaron 92 muestras en las que se midió la [Chl-*a*]. Además, para una mejor interpretación de los resultados se tomaron datos de conductividad y profundidad del disco de Secchi en campo y se recopilaron datos hidrológicos, de precipitación y viento. Los resultados de la validación fueron muy buenos puesto que con la correlación lineal entre datos de campo y estimados se obtuvo una R = 0.8, lo que demuestra la robustez del algoritmo empleado. De la interpretación de los mapas temáticos se pudo extraer que la evolución temporal de la [Chl-*a*] sigue un patrón bimodal anual, donde la disminución de la [Chl-*a*] está determinada bien por un aumento notable de la renovación del agua del lago o por una extinción de los nutrientes disponibles debido a un crecimiento excesivo del fitoplancton.*

Palabras clave: *eutrofización, calidad del agua, gestión hídrica, clorofila-*a*, teledetección*

INTRODUCTION

When considering ecological water quality, eutrophication is currently the main problem in both inland waters (lakes, reservoirs, etc.) and transition and coastal waters of Europe. And that is the greatest threat to biodiversity (Watt *et al.*, 2007) and to an optimal ecologic state in surface waters (CIS, 2005).

One of the main biological parameters used in water ecology and management is the chlorophyll-*a*, because its concentration in the water provides an approximation on phytoplankton biomass (primary producers). Phytoplankton is the group of organisms responding first to nutrient enrichment, with an excessive growth due to their high rates of replication (e. g. Reynolds, 2006). The increase in phytoplankton reflects symptomatic signs of alteration in both the nutrient cycles and the structure of the trophic network, both related to eutrophication.

In Albufera of València, eutrophication is an old problem given that, following intense eutrophication processes throughout the 1960s, the system shifted from a clear into a turbid stable state which was consolidated by the virtual disappearance of macrophytes in the early 1970s (Vicente & Miracle, 1992).

Despite all the efforts to reverse the current state of Albufera, the pressure on this system is increasing by the growth of population in its area of influence (waters with a high load of nutrients) and, in addition, the effects of climate change (reduction of precipitation and increase in evaporation). This situation highlights the importance of constant monitoring of this water mass, even though limnological characteristics and its state have been studied in many works. In this sense, remote sensing is an excellent tool for continuous monitoring of the water mass state.

Although remote sensing on freshwater systems has traditionally been limited by the spatial and radiometric resolution of sensors, its use for the study of the properties and processes of freshwater ecosystems has seen an increase in the last years (Hestir *et al.*, 2015). This increase in the use of remote sensing has also been observed in our study area, where in recent years several studies have been carried out with low and high resolution sensors, both spatial and radiometric: Delegido *et al.*, 2014 and Doña *et al.*, 2014 and 2015.

The European Space Agency (ESA) has just deployed a new Earth Observation Satellites, the Sentinel-2 (S2). The mission started in 2015 and is composed of two satellites, called S2A and S2B, making it an exceptional tool for intensify-

Table 1. Features of the sensors and satellites used in recent remote sensing studies in Albufera of València. *Características de los satelites y sensores utilizados en estudios recientes de teledetección en la Albufera de València.*

Sensor	Bandwidth (nm)	Spatial resolution (m)	Temporal resolution (days)
Landsat-TM/ETM+	> 20	> 30	16
MODIS-TERRA	20	500	1
Deimos-1	>60	22	3
CHRIS/PROBA	5-15	18-36	7
EEI-HICO	5.7	100	3
Sentinel-MSI	> 20	> 10	5

ing studies on water bodies with a spatial resolution (10 m) and a temporary frequency (5 days) unthinkable so far in free and open-access imagery. In Table 1 we can see a comparison between this sensor and those used in the last studies carried out in the Albufera of València.

In 2016, the ESAQS project (Ecological Status of Aquatic Systems with Sentinel Satellites) started, aiming to develop and validate algorithms to estimate ecological quality indicators in inland and coastal waters using the new generation of European Earth Observation satellites: Sentinel-2 and Sentinel-3. The ESAQS project aspires to establish a protocol for regular and frequent monitoring of the ecological status of various reservoirs and lakes in the Valencian region, including the Albufera of València.

In this work we will focus on the study of the temporal and spatial evolution of chlorophyll *a* concentration [Chl-*a*] throughout years 2016 and 2017, in order to show the validity of a proposed algorithm for Sentinel-2 images and an operative method for the multitemporal study in the Albufera of València.

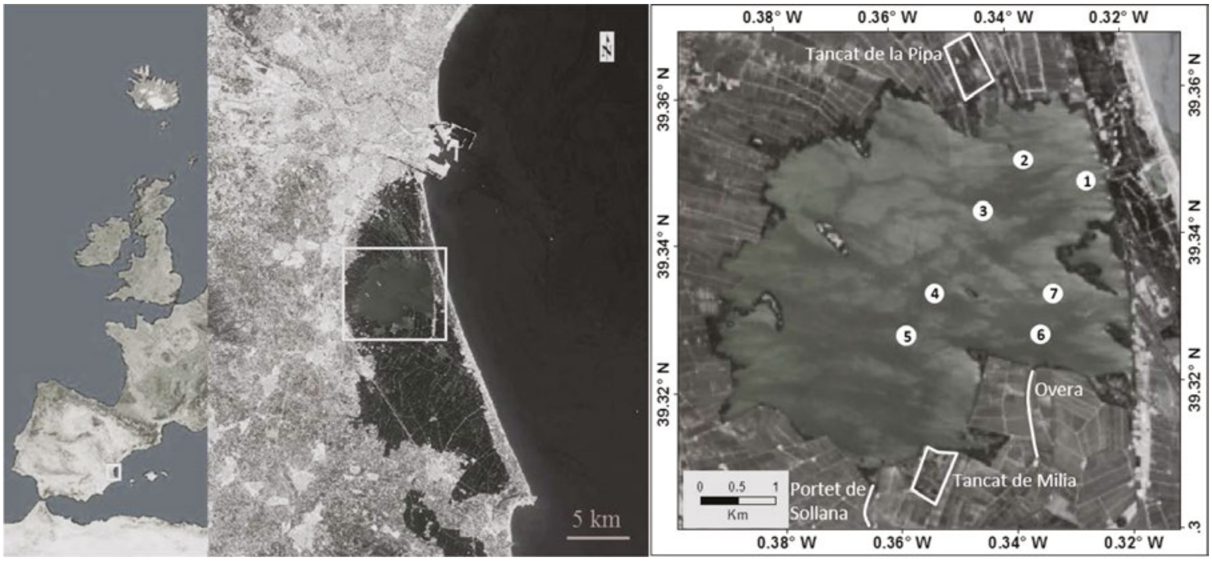

Figure 1. Study site, Albufera of València and sampling locations. *Lugar de estudio, Albufera de València y puntos de muestreo.*

MATERIAL AND METHODS

Study area

Albufera of València, a Mediterranean oligohaline (1-2 ‰) shallow lake (1-3 m), situated 15 km south of the city of València (Spain) (Fig. 1) is the largest natural lake of the Iberian Peninsula. It is considered *a* hypertrophic lake, with average annual chlorophyll a levels of 167 µg/L (4 – 322 µg/L), Secchi disk depth of 0.34 m (0.18 – 1.0 m), total phosphorus of 155 µg/L (41 – 247 µg/L) and total nitrogen of 3.9 mg/L (1.8 – 6.6 mg/L) (Onandia *et al.*, 2015).

Albufera of València hydrology is regulated by the local water council to meet the needs of surrounding agricultural lands, mainly rice paddies. Its outflow to the Mediterranean Sea is controlled by sluicegates located on outlet canals. From November to the beginning of January, the sluicegates are closed, the unsown rice fields remain flooded (the local term for it is *Perellonada*) and the water level of the lagoon is about 20-30 cm above normal. During January and February, there is a period of higher water renewal; flooded rice fields are emptied, water flows out to the sea through the opened sluicegates for that purpose and the level of the lagoon drops. Rice fields remain dry until the beginning of May when they are flooded again for cultivation. During rice planting, the sluicegates are partially closed to maintain a mean water flow and a mean water level (Miracle & Sahuquillo, 2002).

Remote sensing images. Sentinel-2

The ESA S2 mission is a two satellite constellation: S2A (launch date: June 23, 2015) and S2B (launch date: March 7, 2017). Each satellite has a sensor, the MSI, which measures the Earth's reflected radiance in 13 spectral bands from visible to VNIR and SWIR, with a spatial resolution of 10, 20 and 60 m (ESA, 2012). Table 2 shows the most relevant information about the MSI bands and her spatial resolution.

With its 13 spectral bands, 290 km swath width and high revisit frequency (5 days revisit at the Equator), Sentinel-2 supports a wide range

Table 2. Spectral information and spatial resolution of Sentinel-2 A and B for each band (ESA, 2012). *Información espectral y resolución espacial de las bandas de Sentinel-2 A y B (ESA, 2012).*

	S2A		S2B		
Band Number	**Central wavelength (nm)**	**Bandwidth (nm)**	**Central wavelength (nm)**	**Bandwidth (nm)**	**Spatial resolution (m)**
1	443.9	27	442.3	45	60
2	496.6	98	492.1	98	10
3	560.0	45	559	46	10
4	664.5	38	665	39	10
5	703.9	19	703.8	20	20
6	740.2	18	739.1	18	20
7	782.5	28	779.7	28	20
8	835.1	145	833	133	10
8a	864.8	33	864	32	20
9	945.0	26	943.2	27	60
10	1373.5	75	1376.9	76	60
11	1613.7	143	1610.4	141	20
12	2202.4	242	2185.7	238	20

of land studies and programs, and reduces the time required to build an European cloud-free image archive (ESA, 2012). The mission was initially optimized for studies on vegetation, urban planning and terrestrial ecosystems, but the inclusion of new bands in the *red-edge* (the limit of red and infrared spectral regions), its radiometric quality and its high spatial resolution, has proven its usefulness for the study of inland waters (Soria *et al.*, 2017).

Images used in this study were downloaded from the ESA archives and the dataset consists of Sentinel 2 A and B imagery. Software SNAP version 5 (Brockmann Consult) was used for image processing. All images were downloaded in L1C product in order to use the same atmospheric correction for all of them, by means of the Sen2Cor processor. This processor was designed for vegetation and land, but provides good results in eutrophic waters (Soria *et al.*, 2017; Ruescas *et al.*, 2016).

As the algorithm used in the analysis -described below- uses bands of different spatial resolution, the images have previously been resampled at 20 m with SNAP interpolation tools.

Methods

Throughout 2016-2017 Sentinel 2 was able to take 81 images in our study area (62 by S2A and 19 by S2B), but cloud coverage has only allowed to use 40 remote sensing images to observe the spatial and temporal variation of [Chl-*a*] along this period. The time window between image acquisition and field campaigns was extended to 3 days following the methodology of Kutser (2012). We used 17 images that were close to campaign data: 7 were coincident, 3 with one day difference, 5 with two days difference and only 2 with three days difference.

The algorithm used for estimated [Chl-*a*] from S2 images to obtain thematic maps is an adaptation of TBDO, triband model of Dall'Olmo *et al.* (2003), developed for the Albufera of València in Soria *et al.* (2017) and Pereira-Sandoval *et al.* (2018):

$$TBDO = R(740) \times \left(\frac{1}{R(665)} - \frac{1}{R(705)}\right) \qquad (1)$$

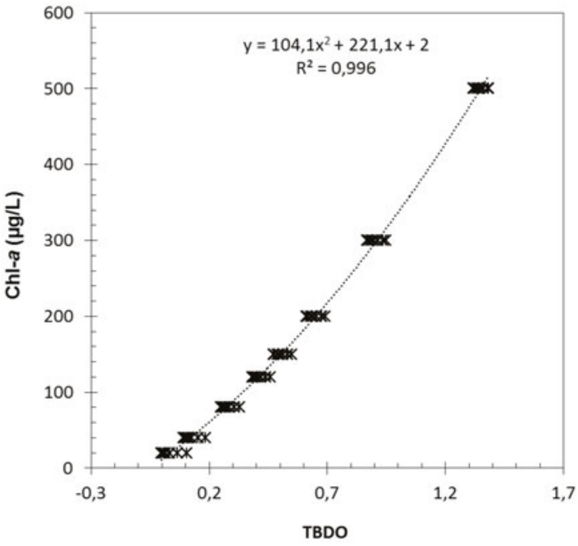

Figure 2. Triband model, TBDO, calibration (Soria *et al.*, 2017; Pereira-Sandoval *et al.*, 2018). *Calibración del modelo tribanda, TBDO (Soria* et al.*, 2017; Pereira-Sandoval* et al.*, 2018).*

Figure 2 shows the relationship between [Chl-*a*] and triband model ($R^2 = 0.996$) and the corresponding algorithm, a second degree polynomial fit intuited from the data structure and avoiding negative predictions of the model due to low chlorophyll values, which would occur with a simpler lineal fit. This calibration was performed with a simulated S2 reflectivity base with a radiative transfer model, Hydrolight (HE5), for a range of [Chl-*a*] between 20 and 500 µg/L.

In summary, the equation applied on the S2 images to obtain [Chl-*a*] was:

$$[\text{Chl-}a]\ (\mu g/L) = 104.1\ TBDO^2 + 221.1\ TBDO + 2 \qquad (2)$$

TBDO index has previously been obtained using equation (1).

Field campaigns

To validate the estimated data, water samples were collected at seven different points within the lake (Fig. 1), but we did not always take all the points. From January 2016 to November 2017, a total of 18 field campaigns were completed and 92 georeferenced samples were collected.

Conductivity and Secchi disk depth were measured *in situ*, and [Chl-*a*] was measured in laboratory using a spectrophotometer. Samples were filtered through 0.4-0.6 µm GF/F glass fiber filters, extracted according to standard methods (Shoaf & Lium, 1976) and data was obtained using the calculation methods by Jeffrey & Humphrey (1975). Meteorological information was provided by the Tancat de la Pipa weather station, situated close to the northern lake shore.

Statistical analyses

To peruse [Chl-*a*] data of the thematic maps presented in this work, obtained by applying equation (2) to S2 images, a boxplot with data on the entire surface of the lake for each image was created. This enables us to see the data dispersion on each one of them, and observe variations depending on the levels of [Chl-*a*] in the lake.

For the comparison between estimated data and field data, its adjustment to a linear regression was checked and the root-mean-square error (RMSE) and percentage of mean absolute error (MAE) were calculated. Additionally, to see whether differences between the values provided by the different monitoring points were statistically significant the Student t-test was applied.

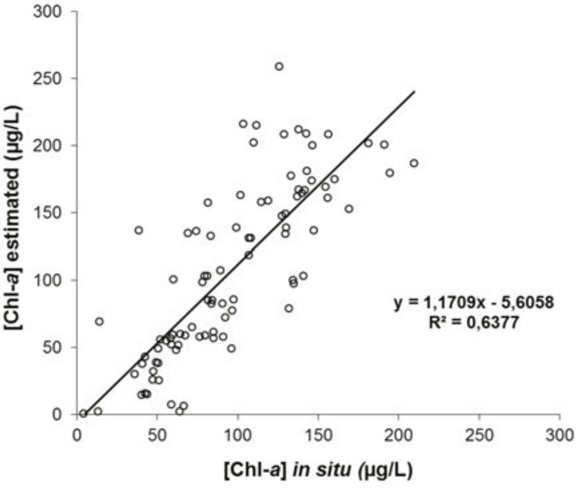

Figure 3. Algorithm validation with field data. *Validación del algoritmo con datos de campo.*

RESULTS

Field data

During the two years of study, a total of 92 samples were collected on 18 different dates. From these samples we will show the mean, the maximum value and the minimum value for each of the measured variables.

The average of the results of [Chl-*a*] was 97 µg/L, ranging between 209 µg/L registered at the sampling point 2 (North) during October 2017, and the 4.5 µg/L registered at the sampling point 6 (South) during April 2017. These values show the great spatial and temporal variability occurring within the lake. To a lesser extent, the Secchi disk depth data also shows this variability, with the maximum value of 0.62 m recorded in April 2017 in the South and the minimum value of 0.2 m observed in sampling points 4 and 5 in January 2016; the average was 0.3 m. Regarding conductivity, the mean during the two years of sampling was 2137 µS/cm, with a maximum value of 3120 µS/cm registered at point 1 (East) in September 2017, whereas the minimum value was at sampling point 7 in January 2017.

Algorithm validation

In order to validate the applied algorithm (equation 2), the linear regression method was used with the field data and values estimated by the algorithm from the S2 images (Fig. 3). We obtain an R of 0.80 for a total of 92 samples, which places us at a degree of significance lower than 0.001. In addition, the slope value is very close to 1, with an intercept value close to 0 considering that we work with values between 4 and 200 µg/L, thus ratifying the high predictive capacity of the algorithm used.

Regarding the calculated errors, the RMSE reached a value of 40 µg/L and the percentage of MAE was 9.6 %.

Image output

By applying equation 2 to S2 images we obtain maps of [Chl-*a*] that allow us to observe its temporal and spatial evolution. Out of 40 images

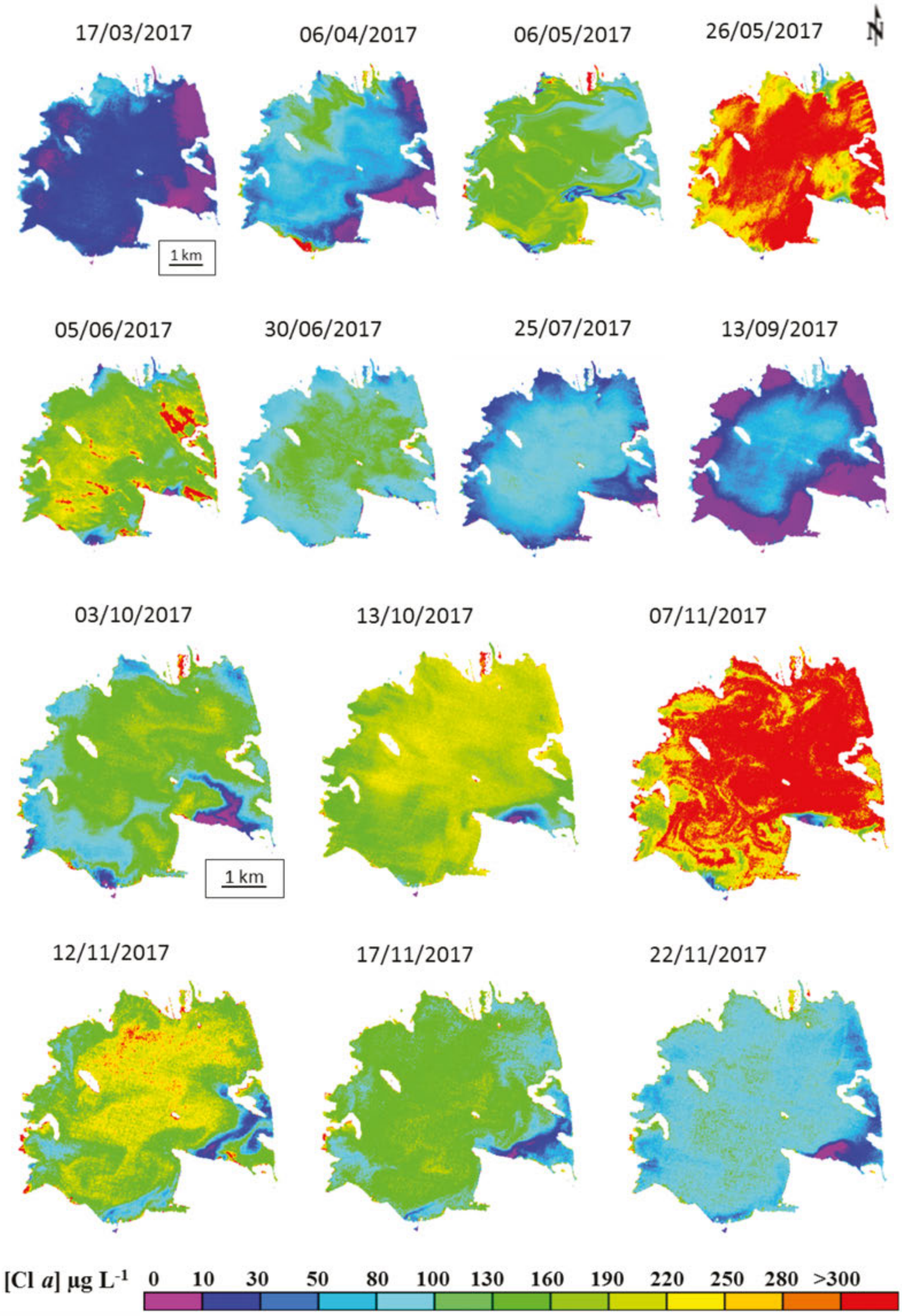

Figure 4. Temporal evolution of [Chl-*a*] in Albufera of València for the year 2017. *Evolución temporal de la [Chl-*a*] en la Albufera para el año 2017.*

processed for this work, in Figure 4 we present only 14, those that clearly illustrate changes in [Chl-*a*]. The sequence of images selected shows the annual cycle of [Chl-*a*] throughout 2017. Images in the first row show the increase of [Chl-*a*], from the low concentrations in March to those highest in late May - it begins in the north, then expands to the south and finally spreads to the east. In the following months, [Chl-*a*] decreases progressively from the shore towards the center of the lake until reaching the lowest values at the end of summer. In the third row, an increase of [Chl-*a*] is observed from the center of the lake towards the shore, until reaching a maximum of [Chl-*a*] in November, the autumn maximum. Ultimately, in the last three images of the sequence, we observe how [Chl-*a*] decreases from the coast towards the center of the lake. Throughout this sequence it is evident that the values of [Chl-*a*] around sampling point 6 (South) are consistently much lower than those in the rest of the lake, due to the water intake going through the *sèquia* of Overa (a man-made irrigation canal) – this is true throughout the year except in the summer months.

The [Chl-*a*] data on the entire surface of the lake of the thematic maps are summarized graphically in Figure 5, where it is observed that data dispersion is directly proportional to the median, that is to say, when lake concentration is greater the data dispersion is at its highest. In this graph also we can clearly see the bimodal behavior of [Chl-*a*] throughout the year.

[Chl-*a*] *in situ* vs. [Chl-*a*] estimated

In addition to the linear regression, to reinforce the good connection between the field data and the estimated data, the temporary evolution of [Chl-*a*] (*in situ* and estimated) has been represented in Figure 6 for the two years of study. For the field data we have used the mean chlorophyll values for each sampling and for the estimated data the mean of the points corresponding to the sampling sites nearest the sampling day (Fig. 6 A). As we can observe, both field and estimated data describe the same variation pattern of [Chl-*a*] over the two years.

In Figure 6, we also plot the values of [Chl-*a*] in field and estimated data for the points located at the North (Fig. 6 B) and South (Fig. 6 C) of the lake. Those points registered the maximum and minimum values respectively, and the difference between its [Chl-*a*] values have proven to be statistically significant (t = 2.202; p = 0.035) according to the Student t-test. Such difference was also significant between point 6 (South) and point 1 (East): t = -2.429; p = 0.021. That exemplifies the differences between the northeastern and southern parts of the lake. Thus, in Figure 6 we can also observe how the algorithm responds appropriately for high and low [Chl-*a*].

Chlorophyll evolution

In order to be able to represent [Chl-*a*] evolution with greater precision, the means for all the

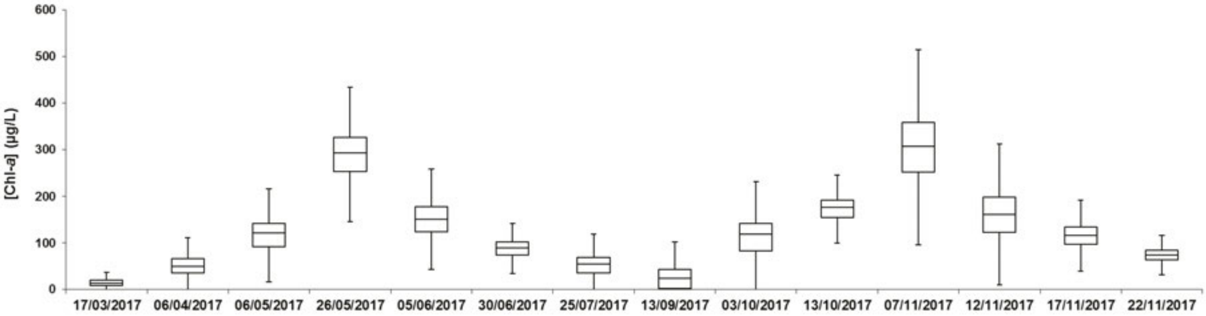

Figure 5. Boxplot of the values range estimated for the images shown in figure 4. For each image, the box bounds the interquartile range (IQR; 25–75 percentile), the horizontal line inside the box indicates the median, and whiskers are 1.5*IQR. *Gráfico de cajas del rango de valores estimado para cada imagen mostrada en la figura 4. Para cada imagen, la caja limita el rango intercuartil (IQR; percentil 25-75), la línea horizontal dentro de la caja indica la mediana y los bigotes son el 1.5*IQR.*

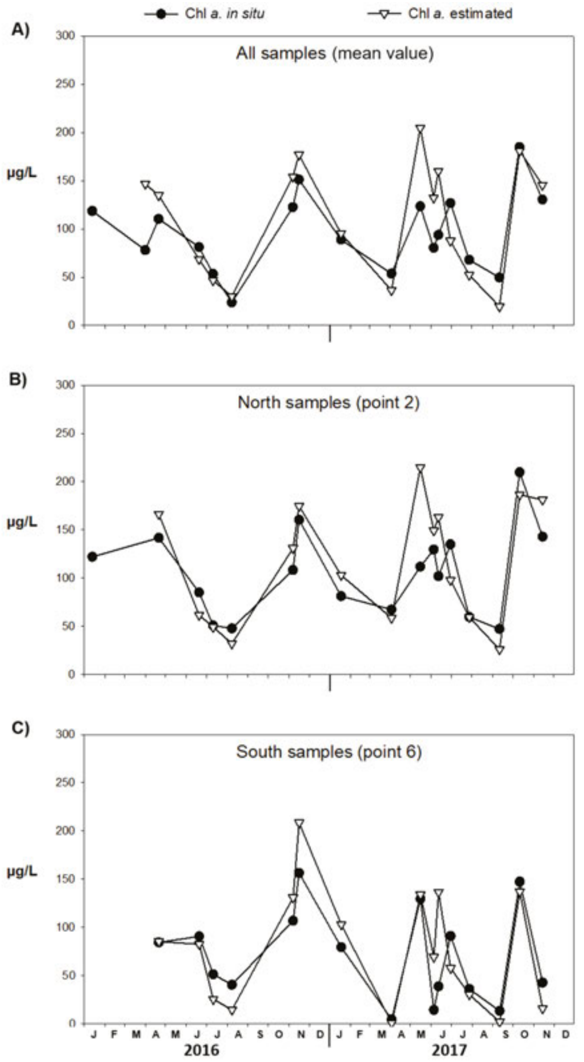

Figure 6. Comparison between *in situ* and estimated [Chl-*a*] using on the one hand the mean of all samples (A) and on the other hand the values of North (B) and South (C) sampling points separately. *Comparativa entre la [Chl-*a*] in situ y estimada utilizando por un lado la media de todas las muestras (A) y por otro lado los valores de los puntos de muestreo Norte (B) y Sur (C) por separado.*

[Chl-*a*] values on the lake surface were calculated for each one of the 40 processed images. This evolution is represented in Figure 7 along with the conductivity and Secchi disk depths of the field values, and meteorological information on precipitation. [Chl-*a*] evolution (Fig. 7A) over the two years registers two minima and two maxima, very marked annual peaks - minima are registered one in March (beginning of spring), the other at the end of August and the beginning of September (end of summer); peaks are seen in May (end of spring) and November (end of autumn). Looking closely at the two minima, we can see at graph C that they coincide with moments of greatest water renewal (if we exclude the winter period).

Regarding conductivity (Fig. 7B), it stays above 1500 µS/cm throughout the year, with maximum values recorded during the summer months. At the beginning of 2017 there is a decrease in conductivity, coinciding with a period of heavy rains occurred at the end of 2016 and the beginning of 2017 (Fig. 7C), happening at the time of greatest water renewal of the lake.

In the Secchi disk depths evolution for 2017 (Fig. 7D), the two peaks with greater transparency coincide with the moments of minor [Chl-*a*], however, in 2016 there are no major variations.

DISCUSSION

Regarding the algorithm used to estimate the [Chl-*a*] values from S2 images, even though the RMSE calculated with the data in our study is much higher than that calculated by Soria *et al.* (2017) of only 15.7 µg/L, the good coefficient of correlation between the field and estimated data, the low percentage of MAE (9.6 %) and the correspondence in the temporary variation between *in situ* and estimated data show that the algorithm used delivers very good matching results for the water body studied. Therefore, this algorithm would not require major optimization to carry out accurate estimates of [Chl-*a*] in Albufera of València, a very variable water body as regards to [Chl-*a*].

If we look at the thematic maps obtained (Fig. 4), the annual evolution of the [Chl-*a*] registers two minima and two very conspicuous maxima. This depends greatly on the sampling scheme but a bimodal pattern arises and this is thought to be caused by an assortment of natural and human factors. In the description of the study site we have mentioned that in Albufera of València water system management, human hand plays a determinative role.

The first [Chl-*a*] minimum is recorded around March coinciding with an increase in the lake's

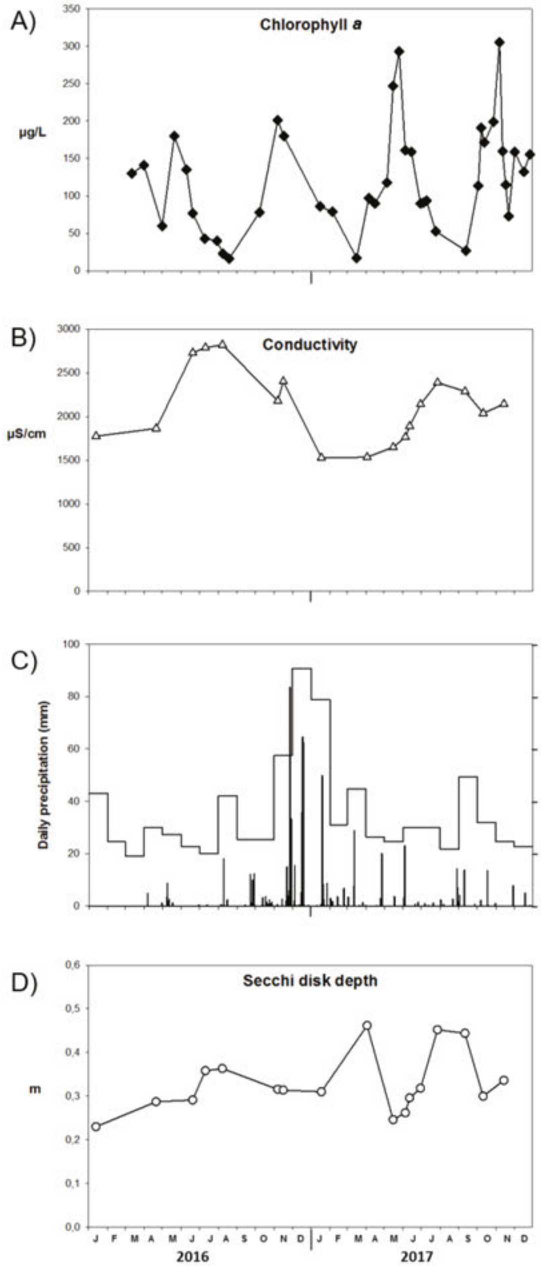

Figure 7. Evolution of [Chl-*a*] using the mean values on the entire water surface from estimated values (A), the mean of the field data for conductivity (B) and Secchi disk depth (D), hydrological information for water renewal (C-coninuous line) and meteorological information for precipitation data (C-bars). *Evolución de la [Chl-*a*] utilizando los datos estimados para toda la superficie de agua (A), la media de los datos de campo para la conductividad (B) y la profundidad del disco de Secchi (D), información hidrológica para la renovación del agua (C-línea continua) e información meteorológica para los datos de precipitación (C-barras).*

water renewal due to water coming from the rice paddies that have been purposefully flooded in winter. After some weeks, the [Chl-*a*] increases remarkably until the first annual maximum that occurs at the end of May. Then there is a sharp decline in the summer period that evidences a decline in primary production motivated by nutrient depletion after the typical production peak in late spring (Russel-Hunter, 1970). This second decline in [Chl-*a*] due to nutrient depletion, is facilitated by an increase in the renewal of the lake's water caused by the draining of the rice paddies previous to the crop harvest period. Lastly, in November, the second yearly maximum of [Chl-*a*] occurs, caused by nutrients entering the system along with the water coming from crop fields. Once nutrients are depleted, [Chl-*a*] stabilizes through the winter.

Temporal sequence variations are not the only information gleaned, the images also highlight the spatial variation of [Chl-*a*]. The differences in the increase of [Chl-*a*] in the spring with respect to the increase in autumn after the two annual minima could be explained by the factors causing the decrease of the [Chl-*a*]. In March the decrease is due to a strong renovation of the lake's water by the draining of the flooded fields, the water within the lake is shifted to the sea, hence the increase of [Chl-*a*] would follow that same trend. However, at the end of the summer the renovation is not of such magnitude and the decrease of [Chl-*a*] is due to the depletion of nutrients, depleted first in the areas where there is a smaller volume of water, that is, by the lake shore, and then spreading towards the center of the lake, so the increase of [Chl-*a*] would follow the opposite route.

Within this spatial heterogeneity, thanks to variations in [Chl-*a*], we can indentify areas of worse or better quality water. We would like to note an important entry point of better quality water at the mouth of the *Overa* irrigation canal, just south where sampling point 6 is located. Thanks to S2 images the water inflow can be observed as well as the behavior of the plume that forms within the lake - that would not be possible highlight with current field sampling techniques. If we follow the dynamics of this better quality water plume, it generally travels eastwards to the watercourses linking the lake with the sea, except when winds at speeds above 20 km/h cause that plume to shift in other directions. Current situation indicates that, in general, water entering through *Overa* only renews the southeastern sector of the lake with an 11-day renovation period (Soria & Vicente, 2002), but this water could make a greater contribution to the lake renovation if we would move the entry point to the southwest as much as possible, thus impacting a sector that has a 53-day renovation period (Soria & Vicente, 2002). Taking into account the connection between irrigation canals, water coming from Overa could be diverted through Campets irrigation canal and then through Portet de Sollana canal so that it finally enters into the southwest part of the lake, near to the Tancat de Milia, a green filter created to minimize the nutrients in the treated water coming out of the Albufera-Sud plant.

If we compare the evolution of the parameters considered with respect to previous studies, the Secchi disk depth values and conductivity are similar to those in earlier studies such as those presented in Soria *et al.* (1987). On the other hand, [Chl-*a*], despite having fluctuations similar to the 1998-2006 period studied in Romo *et al.* (2008), now presents greater amplitudes because decreases of [Chl-*a*] reach lower values, indicating a tendency towards an improvement in the ecological status of the lake.

CONCLUSIONS

In summary, this study shows that the algorithm to estimate the [Chl-*a*] from S2 images is consistent and suitable for use within a protocol whose main purpose aims to monitor Albufera of València's ecological status.

Arguably, the results obtained are dependent on the sample scheme, but an annual bimodal tendency is deduced from the monitoring carried out throughout the period. The decrease of [Chl-*a*] is determined either by a marked increase in the water renewal of the lake or by the depletion of nutrients. As a continuous contribution of quality water seems increasingly unlikely and utopic, we suggest focusing on improving water quality currently entering into the system so as to diminish the peaks of spring and autumn. In this sense, we think certain actions would reduce nutrient inputs and improve water renovation. Such actions might be (1) starting the tertiary treatment in sewage treatment plants draining into the canals leading to the lake, (2) making the green filter at Tancat de Milia effective, (3) encouraging organic farming inside the Albufera of València Natural Park and (4) the efficient management of water contributions to promote the renewal of, especially, the western part of the lake.

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Determination of the Trophic State Index (TSI) using remote sensing, bathymetric survey and empirical data in a tropical reservoir

Iris Amati Martins[1,*], Danielle Fein[2], Marcelo Luiz Martins Pompêo[1] and Marisa Dantas Bitencourt[1]

[1] Department of Ecology, University of São Paulo, Brazil. Rua do Matão, trav. 14, nº 321, Cidade Universitária, ZIP code: 05508-090, São Paulo - SP, Brazil.
[2] Departament of Biology, FMU Education Group - Laureate International Universities, Brazil. Rua Taguá, nº 150, Liberdade, ZIP code: 01508-010, São Paulo - SP, Brazil.

[*] Corresponding author: imartins@ib.usp.br

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ABSTRACT

Determination of the Trophic State Index (TSI) using remote sensing, bathymetric survey and empirical data in a tropical reservoir

The reservoirs are important freshwater reserves that suffer with domestic and industrial wastes, causing qualitative and quantitative changes in their physicochemical composition, as well as causing fauna and flora impacts. One of the consequences is eutrophication, a process characterized by increased concentration of nutrients in aquatic ecosystems, mainly phosphorus and nitrogen. In order to classify waterbodies according to their trophic degrees, chlorophyll-*a* and phosphorus concentration data are usually obtained in water samples, feeding an equation to obtain the Trophic State Index (TSI). The method used at the present work aim to determine the TSI based on remote sensing (RS), by using the ratio between the near-infrared (NIR) and red (RED) images to infer the chlorophyll-a concentration in wet and dry seasons. This method allows a synoptic vision of the reservoir, providing support for decision-making process at low cost, especially when a large amount of data is available and/or generated, increasing quality in consonance with technological development. However, we need to consider the influence of the bottom of shallow areas when using orbital images to evaluate the waterbodies conditions, since the emerging radiation of the euphotic zone is responsible for the results reliability. Thus, it is necessary the exclusion of shallow areas, which may compromise the spectral response of the satellite images due to the bottom effects, before the development of the TSI RS Map. We created the TSI RS Maps (observed) and then we validated it with the Empirical TSI Maps (expected), obtained in 28 geo-coded points along the riverine-transition-lacustrine gradient, both for wet and dry season. We performed the chi-square test that shows how much difference exists between the observed and the expected. Both seasons presented $x^2 = 1$, df = 27, with critical value (0.005) of 49.64. Therefore, we found no differences between the observed and the expected. Our results reinforce the potentiality of the RS to perform the TSI favoring the identification of eutrophic areas in inland water systems with low turbidity.

Key words: limnology, chlorophyll-*α*, phosphorus, Secchi depth, satellite images, euphotic zone, bottom effects, cascade reservoirs, inland water

RESUMO

Determinação do Índice de Estado Trófico (IET) por meio de Sensoriamento Remoto, Levantamento Batimétrico e Dados Empíricos em um Reservatório Tropical

*Os reservatórios são importantes reservas de água doce que sofrem com despejos de esgotos domésticos e industriais que causam mudanças quantitativas e qualitativas na sua composição físico-química, além de causar impactos na fauna e na flora. Uma das consequências é a eutrofização, processo que se caracteriza pelo aumento na concentração de nutrientes no ecossistema aquático, principalmente fósforo e nitrogênio. A concentração de clorofila-*a *e de fósforo são obtidas das amostras da água para alimentar as equações de Índice de Estado Trófico (IET). Os métodos usados no presente trabalho visam determinar o grau de trofia utilizando o sensoriamento remoto orbital (SR), que usa a razão entre as imagens das faixas do infravermelho*

(IVP) próximo e do vermelho (VERM) para inferir a concentração de clorofila-a, bem como a variação do IET, no período seco e chuvoso. Este método permite uma visão sinóptica que possibilita um suporte para tomada de decisão com baixo custo, especialmente quando um grande volume de dados é disponibilizado e/ou gerado, ampliando assim a qualidade em função do desenvolvimento tecnológico. Contudo, é necessário considerar a influência do fundo dos corpos d'água quando imagens de satélite são utilizadas, uma vez que a radiação emergente da zona eufótica é que dá respaldo aos resultados. Desde modo, nós excluímos as áreas rasas que pudessem comprometer a resposta espectral das imagens de satélite devido ao efeito do fundo, antes de serem usadas para produzir o Mapa de IET SR. Nós criamos o mapa de IET SR (observado) e então o validamos pelo Mapa de IET Empírico (esperado), obtido por meio de análises químicas realizadas na água em 28 pontos georreferenciados ao longo do gradiente lacustre-fluvial, ambos para o período seco e chuvoso. Nós realizamos o teste chi-quadrado que apresenta quanta diferença existe entre o observado e o esperado. Ambos períodos apresentaram $x^2 = 1$, *gl = 27, com valores críticos (0.005) de 49.64. Desse modo, não encontramos diferenças entre o observado e o esperado. Nossos resultados reforçam a potencialidade do SR na obtenção do IET, favorecendo a identificação de áreas eutróficas em sistemas de águas continentais que apresentam baixa turbidez.*

Palavras chave: *limnologia, clorofila-α, fósforo, profundidade de Secchi, imagens de satélite, zona eufótica, efeito de fundo, reservatórios em cascata, águas continentais*

INTRODUCTION

The planet Earth has two thirds of its surface covered by water, however more than 98 % of available water is salty and only 2 % is freshwater (low salinity), which more than 68 % are distributed in glaciers, 29 % are in deep groundwater reservoirs that are difficult to access, and only 1.2 % is available in rivers and lakes (Senra, 2001). In Brazil, freshwater availability can be considered generous, accounting for 12 % of the planet's water reserves (Senra, 2001), with a surface water production of 168 790 m^3/s (Novaes, 2000). According to the São Paulo State Secretary for Water Supply, about 86 % of the territory of São Paulo State is in the Paraná river basin. The public drinking water supply for the Metropolitan Region of São Paulo (MRSP) is a very important issue due to its population of 17.2 million, distributed along 39 municipalities (EMPLASA, 2000). The Cantareira System, or Cantareira Complex, is a water supply system within the MRSP, which comprises five interconnected reservoirs (Cascade Reservoirs), ending (downstream) in the Juqueri River Reservoir (also known as Paiva Castro Reservoir) and Águas Claras Reservoir, before reach the Water Treatment Plant (WTP Guaraú).

The eutrophication comprises a suite of symptoms that reservoirs exhibit in response to fertilization with nutrients (Hutchinson, 1973). These symptoms include several physical-chemical and ecological alterations listed in the limnological literature (Straškraba, 1999; Cooke *et al.*, 2005; Jørgensen *et al.*, 2005; Jacoby *et al.*, 2007; Schindler *et al.*, 2008; Tundisi & Tundisi, 2008). The eutrophication arises from the oversupply of nutrients in aquatic ecosystems, mainly phosphorus (P) and nitrogen (N), resulting in increased productivity (Esteves, 2011), which leads to overgrowth of plants and algal blooms that can form masses, floating islands or algal mats that can be displaced by the winds and accumulate on the margins, where its begin to decompose and cause unpleasant smell and production of toxic substances (Branco, 1986). Because of this process, the aquatic ecosystem changes from oligotrophic and mesotrophic to eutrophic or even hypertrophic conditions (Esteves, 2011). To classify the eutrophic state of a reservoir some parameters can be used: total P and total N, chlorophyll-*a* concentration, and Secchi disk depth (Andreoli *et al.*, 2011). It is also very common the use of Trophic State Index (TSI), which is an important indicator in the decision-making process concerning the reservoir management. The Carlson (1977) TSI was adapted by Lamparelli (2004) in order to fulfill tropical specification, evaluating water quality related to nutrient enrichment and its effect on excessive algae growth and increased infestation of aquatic macrophytes. Nowadays it is the index used regularly by the official agencies (CETESB, 2013).

Another method to estimate the TSI is those based on satellite images, which can be of great help for decision makers since it allows a synoptic view of the reservoir trophic state. The first attempts to apply remote sensing (RS) to monitoring the eutrophication process date from the 1970s, but the remote sensors available at the time were not suitable to the complexity of inland waters (George & Malthus, 2001). With the improvement and the popularization of the satellite imagery along the last decades, several other TSIs were proposed (Novo, 2007). Yacobi *et al.* (2011); Gitelson *et al.* (2011), Dall'Olmo & Gitelson (2005), Gons *et al.* (2002), Gitelson & Kondratyev (1991) suggested that red (RED) and near-infrared (NIR) spectral responses can be particularly helpful to estimate chlorophyll-*a* concentration in eutrophic waterbody, where Colored Dissolved Organic Matter (CDOM) are abundant. Duan *et al.* (2007) studied Changan Lake, northeast China, demonstrated the efficiency of the ratio between NIR and RED to estimate chlorophyll-*a* concentration. Novo *et al.* (2013) proposed recently a new TSI using orbital remote sensing in Ibitinga Reservoir, part of Tietê River dam complex, located in the geographic center of the São Paulo State in the Middle Tietê River Valley. Using Monte Carlo simulation, the authors selected the most suitable model relating to chlorophyll-*α* concentration and simulated TM/Landsat reflectance. The ratio between NIR (TM4) and RED (TM3) provided the best model with a R^2 value of 0.78, enhancing the relationship between chlorophyll-*a* concentration and remotely sensed reflectance, since the high concentration of phytoplankton increases the reflectance in the NIR and decreases the reflectance in the RED. However, it is necessary to consider the effects of the reservoir bottom in shallow areas, which may compromise the information contained in the satellite images, being sure that the emerging radiation used to calculate the TSI comes exclusively from the euphotic zone (Kirk, 2011; Bitencourt & Mantelli, 2015).

As the costs for conventional limnological monitoring (water sampling and laboratory analysis) are high, it is important and often necessary to use monitoring methods that seek to reduce costs, in addition to optimize acquisition results. The use of new data sources and spatial analysis tools, such as RS and geoprocessing, can contribute to reducing costs, since there are satellite images and geoprocessing software packages that are free. The RS also allows an efficient and constant acquiring of data sets along the time and space, contributing with the decision-making process in the water resources management.

The main objective of this paper is to produce a TSI Map using Remote Sensing (observed) according to Novo *et al.* (2013) and validate it with an Empirical TSI Map (expected) performed by using field data (concentrations of chlorophyll-*a* and total P), for both wet and dry seasons in the Paiva Castro Reservoir. To achieve that objective, it was necessary to make some maps using models already presented in the literature and/or to create new other maps. The reservoir bathymetry map, for example, had to be created. Therefore, bathymetric field data should be sampled and analyzed beforehand. Therefore, the flowchart for bathymetry is a brand-new way of obtaining such map and the description step by step can very helpful to further decision makers. Other flowcharts are also presented to help decision makers, but they are not of our complete authorship even though they show the step by step to generate each thematic map used as the basis for development of the TSI maps, and the steps to generate the TSI maps itself. Each step was carefully examined and justified in order to improve confidence. We hope to reinforce the potentiality of the Remote Sensing data (RS) to perform the TSI in tropical reservoirs, also acquiring some important limnologic parameters like chlorophyll-*a* concentration, Secchi depth and euphotic zone extension, in different space and time scales depending of the resolution of the remote sensor.

MATERIALS AND METHODS

Study Area

The Paiva Castro is the fifth downstream reservoir of the Cantareira System just before Águas Claras Reservoir where the water is treated to become drinkable. The System was concluded in 1973 (Figs. 1 and 2). Its watershed comprises 33 715 ha, mainly composed by native forest, herbaceous vegetation and forestry, with little

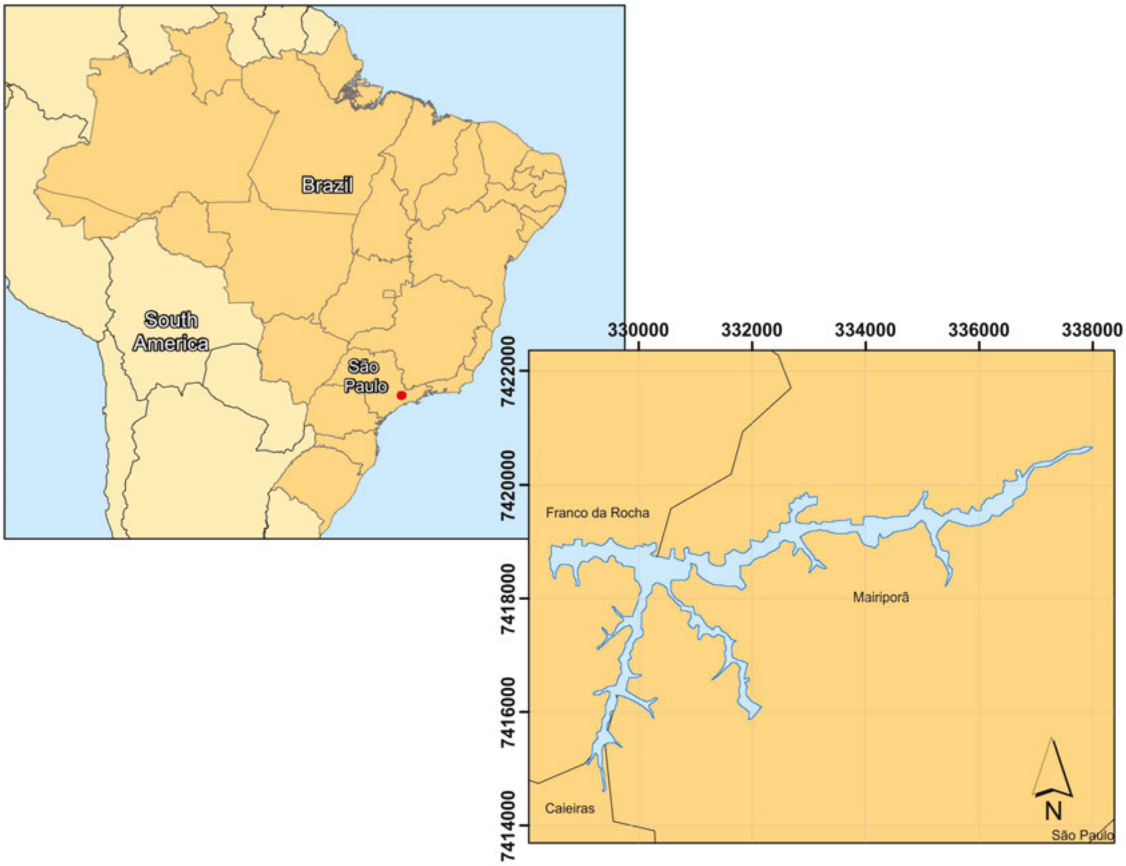

Figure 1. The location of the Paiva Castro Reservoir. *Localização do Reservatório Paiva Castro.*

influence of urban areas. The reservoir contributes with 6.1 % of the whole Cantareira System water production. Its surface area comprises 445 ha and the retention time is 14 days, which characterizes the Paiva Castro Reservoir more lotic than lentic.

Bathymetric Survey

To obtain the bathymetric map of the reservoir we had three days of field work using a marine Geographical Position System (GPSMAP GARMIN 421S) coupled with a transducer to get X and Y coordinates and the reservoir depth. To check error positions, we used a Differential Global Positioning System (DGPS), GNSS Sf3040-Rtk-Sfto receptor. To get Z elevation and to determine and evaluate the errors of X and Y positions, we used the software R (2008). Further analyses were performed in order to obtain the final elevation model.

Remote Sensing (RS)

To develop the Euphotic Zone Map and the TSI RS Map we used Landsat 5 - Thematic Mapper (TM) images from two dates: January 9th and May 1rst 2009, wet and dry season respectively. We selected those dates due to the proximity with the period in which the empirical data sampling was performed and because of the absence of clouds. We obtained the images from the Brazilian National Institute for Space Research (INPE) catalog (INPE, 2016). We used the reservoir polygon obtained through bathymetric survey to constrain the reservoir area in the Landsat images.

Using the software Terrset (Eastman, 2015), we performed the processing of the Landsat images aiming to minimize the Rayleigh scattering effects (atmospheric correction). The method of atmospheric correction was the Total Correction Model (Eastman, 2015), which includes the parameters used in the Dark Object Subtraction Model (DOS) and the Cos (t) Model developed by Chavez (1996). We also performed the radiometric correction aiming to correct the radiometric degradations due to mismatches in the calibration of the detectors and errors in the data transmission, a procedure that ensures that changes in spectral behavior, both in time and in space, are strictly related to the interaction of the electromagnetic radiation with the target, eliminating contributions of the environment in the reflectance of the target (Moreira, 2012).

Empirical Data

We used as empirical data the georeferenced data sampled by Macedo (2011) in 28 points along the riverine-transition-lacustrine gradient of the Paiva Castro Reservoir, at wet season (November 2008) and dry season (June 2009). She determined the total P and chlorophyll-*a* concentration by spectrophotometry (Wetzel & Likens, 1991; Valderrama, 1981), and the Secchi depth by Secchi disk.

Euphotic Zone Mask

The euphotic zone mask aims to constraint the area of analysis in the satellite images avoiding the bottom effects of shallow areas. Thus, we used the ratio between BLUE (TM2) and TM4 images to obtain the Secchi depth (Pereira *et al.*, 2011). The next step was the development of the euphotic zone image based on Esteves (2011), Bukata *et al.* (1995), and Kirk (2011). We performed a logical operation to create a mask that excluded the shallow areas of the reservoir. If the extension of the euphotic zone was equal or more than depth obtained by the bathymetric survey, the area equivalent was excluded. If the extension of the euphotic zone was less than depth, the area equivalent was included, i.e., the area that will be the object to performing TSI RS. We present here a flowchart that shows the step by step to obtain the euphotic zone mask.

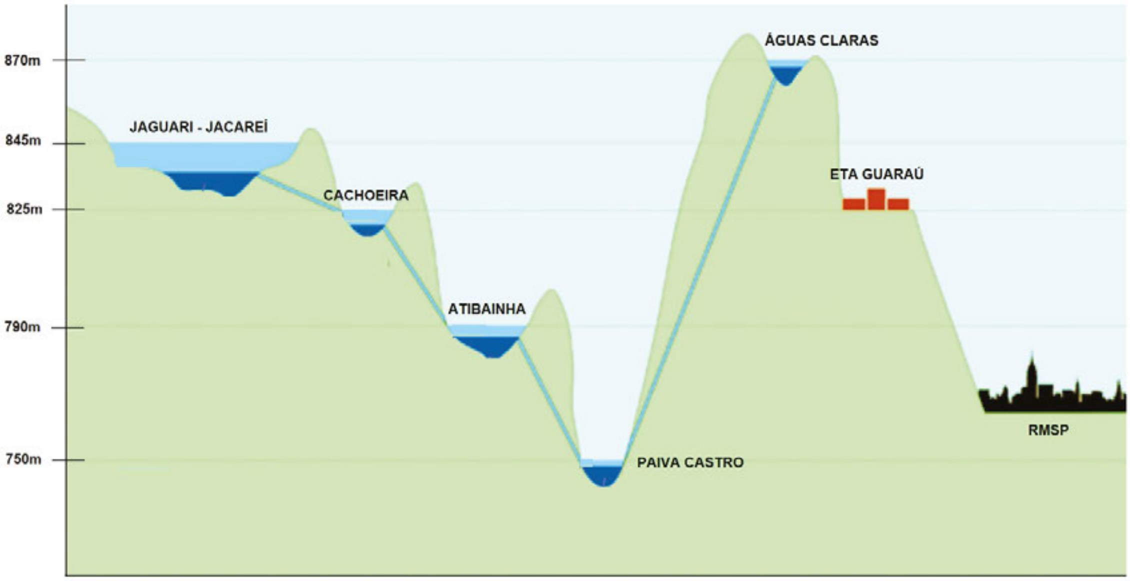

Figure 2. The Cantareira System (adapted from ANA, 2013). ETA: Estação de Tratamento de Água (Water Treatment Plant); RMSP: Região Metropolitana de São Paulo (Metropolitan Area of Sao Paulo). The Y-axis represents the altitude. *O Sistema Cantareira (adaptado de ANA, 2013). ETA: Estação de Tratamento de Água; RMSP: Região Metropolitana de São Paulo. O eixo Y representa a altitude.*

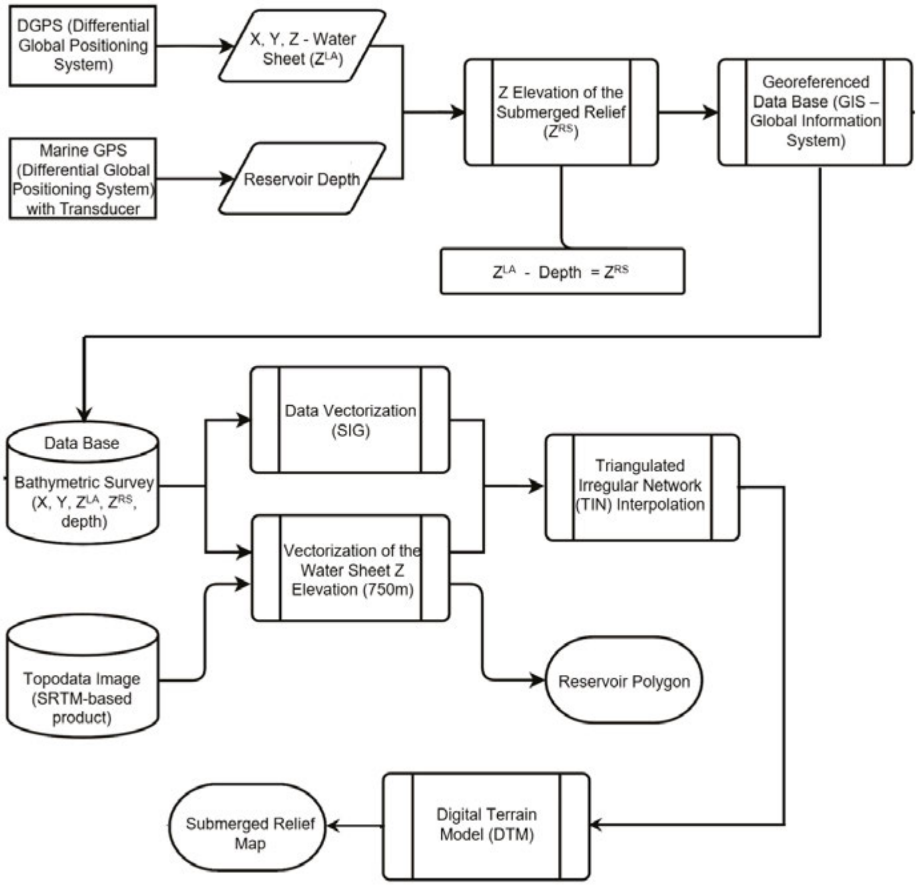

Figure 3. Flowchart showing the steps to perform the bathymetric survey. *Fluxograma apresentando as etapas para o desenvolvimento do levantamento batimétrico.*

Trophic State Index (TSI)

To obtain the TSI Map using RS for wet and dry seasons, we used the TM4 and TM3 images from Landsat 5. We applied the euphotic zone mask to these images to eliminate the shallow areas and consequently the bottom effects within the spectral response registered by satellite images. Therefore, we used the model based on the TM4/TM3 ratio proposed by Novo *et al.* (2013) to obtain the chlorophyll-a concentration. Afterwards, we calculated the Lamparelli (2004) TSI and then we classified the TSI in the following classes: ultraoligotrophic (TSI < 47), oligotrophic (47 < TSI ≤ 52), mesotrophic (52 < TSI ≤ 59), and eutrophic (59 < TSI ≤ 63), producing the TSI RS Maps.

To develop the Empirical TSI Map firstly we calculated the Lamparelli (2004) TSI using the concentrations of chlorophyll-*a* and total P obtained in water samples for both seasons. As the water samples are geocoded, we performed the TIN interpolation of the TSI values and then we converted the resulting polygons into a surface image. Thereafter, we classified the Empirical TSI image in the following classes: ultraoligotrophic (TSI < 47), oligotrophic (47 < TSI ≤ 52), mesotrophic (52 < TSI ≤ 59), and eutrophic (59 < TSI ≤ 63), resulting in an Empirical TSI Maps.

Geostatistical

We validated the TSI RS Maps (observed) toward the Empirical TSI Maps (expected). We performed the validation in two ways: (a) using the chi-square test, that shows how much difference exists between the observed and the expected; (b) using a Contingency Table or Error Matrix, where the columns represent the reference data or the data that is known to be true (empirical), and the rows are the classes that were mapped and generated from the RS data. Doing so, we obtained the Kappa Index in order to evaluate the concordance of the classification (Congalton & Mead, 1991).

RESULTS

The bathymetric survey was performed in four stages: (a) the first stage resulted from three

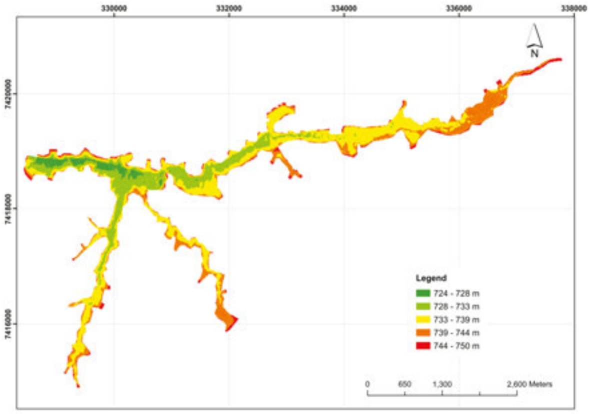

Figure 4. Submerged Relief Map. *Mapa do Relevo Submerso.*

Table 1. Comparison between field values and remote sensing values for Secchi Depht (m) and Euphotic Zone (m). *Comparação entre os valores de campo e os valores obtidos por sensoriamento remoto para a profundidade de Secchi (m) e Zona Eufótica (M).*

ID	UTM Coordinates		Secchi Depht (m)				Euphotic Zone (m)			
	(Datum WGS84, 23K)		Field Values		RS Values		Field Values		RS Values	
	X	Y	Dry	Wet	Dry	Wet	Dry	Wet	Dry	Wet
P2	337360	7420431	1.27	1.20	2.13	2.24	3.42	3.25	5.75	6.05
P3	336906	7420283	1.27	1.20	2.65	2.63	3.42	3.25	7.16	7.10
P4	336669	7419810	1.27	1.20	2.38	2.56	3.42	3.25	6.43	6.91
P6	335866	741950	1.16	1.10	2.58	2.57	3.13	2.97	6.97	6.94
P7	335466	7419404	1.06	1.18	2.65	2.68	2.86	3.19	7.16	7.24
P8	335102	7419506	0.94	0.95	2.65	2.65	2.54	2.57	7.16	7.16
P9	334722	7419326	1.00	1.15	2.65	2.62	2.70	3.11	7.16	7.07
P10	334356	7419371	0.92	1.13	2.64	2.69	2.48	3.05	7.13	7.26
P11	333952	7419253	0.92	1.14	2.65	2.65	2.48	3.08	7.16	7.16
P12	333553	7419297	0.85	1.18	2.66	2.66	2.30	3.19	7.18	7.18
P13	333104	7419243	0.90	1.07	2.63	2.67	2.43	2.89	7.10	7.21
P14	332671	7419204	0.75	1.22	2.65	2.65	2.03	3.29	7.16	7.16
P15	332373	7418951	0.92	1.01	2.64	2.67	2.48	2.73	7.13	7.21
P16	331990	7418795	0.91	1.13	2.63	2.66	2.46	3.05	7.10	7.18
P17	331652	7418499	1.05	0.93	2.65	2.68	2.84	2.51	7.16	7.24
P20	330637	7418484	1.65	1.17	2.65	2.68	4.46	3.16	7.16	7.24
P21	330246	7418640	1.33	1.32	2.64	2.67	3.59	3.56	7.13	7.21
P22	329880	7418714	1.76	1.32	2.64	2.64	4.75	3.56	7.13	7.13
P23	329562	7418891	2.18	1.17	2.64	2.65	5.89	3.16	7.13	7.16
P24	329208	7418849	2.19	1.38	2.65	2.64	5.91	3.73	7.16	7.13
P26	328532	7418942	2.37	1.46	2.65	2.68	6.40	3.94	7.16	7.24
P27	330191	7418257	1.31	1.07	2.64	2.68	3.54	2.89	7.13	7.24
P28	330113	7417901	1.18	1.22	2.64	2.67	3.19	3.29	7.13	7.21
P29	329986	7417565	1.18	1.27	2.28	2.21	3.19	3.43	6.16	5.97
P30	329895	7417203	1.13	1.44	2.59	2.53	3.05	3.89	6.99	6.83
P32	329728	7416921	1.17	1.37	2.65	2.66	3.16	3.70	7.16	7.18
P33	329522	7416068	1.17	1.52	2.68	2.12	3.16	4.10	7.24	5.72

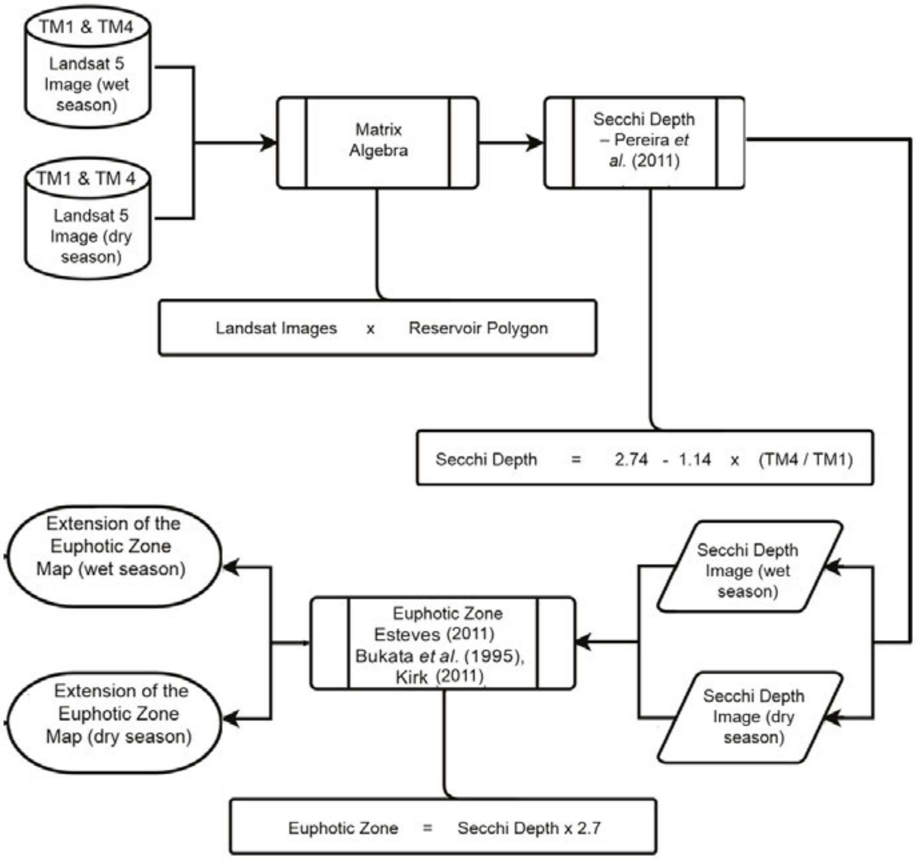

Figure 5. Flowchart showing the steps to perform the Euphotic Zone Map. *Fluxograma apresentando as etapas de desenvolvimento do Mapa de Zona Eufótica.*

days of field work, using a marine GPS with transducer installed in the boat and a DGPS; (b) the second stage was the pre-processing of sampled data, associating the information of the marine GPS with the DGPS and analyzing the difference between the X and Y axes; (c) the third stage comprises the interpolation of the sampling data by using the Triangulated Irregular Network (TIN) and the conversion into surface (Martins, 2017); and (d) the fourth stage occurred during three more days of field work, aiming to complete the bathymetric survey, fulfilling the gaps of the previously survey. Finally, 16 529 points were sampled along the reservoir of approximately $4.49 \cdot 10^6$ m^2. We determined the sample sufficiency with 1 % margin of error and 99 % confidence interval. To perform the sampling, the boat bordered the entire reservoir and then crossed two parallel lines along the reservoir. The next step was to make a zigzag from margin to margin. We recorded the data every 10 m with the boat stopped to obtain information from the marine GPS and DGPS.

The data distribution of the first bathymetric survey allows us to detect that the differences between the marine GPS and DGPS. The results were the constancy between the X and Y axes, except to a few outliers (0.75 % of the surveyed points) that we excluded based on the mean and standard deviation. The creation of the bathymetric function ($Bat_{f(x)} = X - 40$ and $Bat_{f(y)} = Y - 44$), allow us to adjustment of the X and Y axes for further surveys and to model the submerged relief.

After pre-processing the data using bathymetric function, the data were TIN interpolated and converted them into a surface image. At this stage, the information related to the Z elevation value from the reservoir water level was recorded.

The bathymetric survey also allows us to obtain the reservoir polygon, considering the almost constant surface level of 750 m of altitude. That polygon we used to constraint the satellite images to perform all the geospatial analyses. The Figure 3 presents the flowchart of all steps to obtain the bathymetric survey. The Figure 4 reveals the submerged relief map.

The Secchi depth values found using RS were quite homogeneous along de reservoir ranging from 2.6 m to 2.7 m mainly in the central portion of the reservoir and at the border the values were near to 2.0m. We did not observe differences between wet and dry seasons although, the results of the empirical Secchi depth, shows a high amplitude along the reservoir, 17 times higher than RS values in wet season and 9 times higher in dry season (Table 1).

We created the Euphotic Zone Maps using the Secchi depth (SD) images obtained with RS (Fig. 5). We did not observed differences between the wet and dry season in areas that have the extension of the euphotic zone until 3 m. On the other hand, areas with the euphotic zone extension between 4-5 m present an increase of 51.2 % in the dry season, while areas with the euphotic zone extension within the range of 7 m decreases in the dry season (8.1 %)

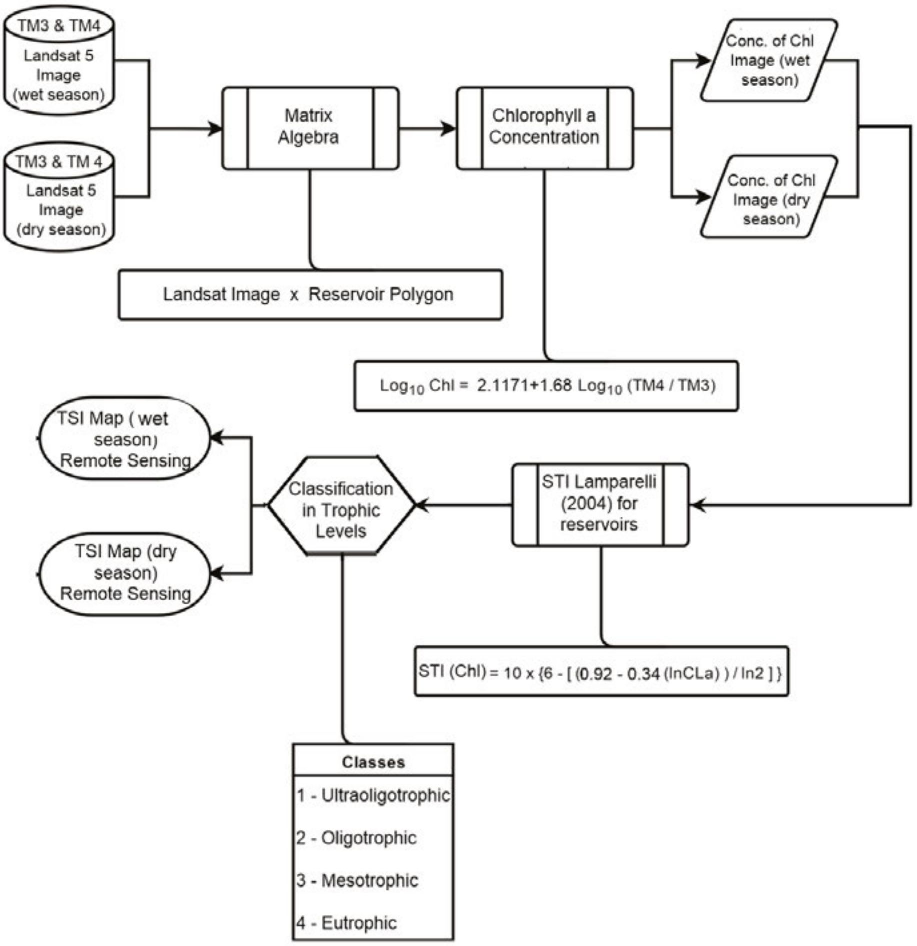

Figure 6. Flowchart showing the steps to perform the TSI Remote Sensing Map. *Fluxograma apresentando as etapas de desenvolvimento do Mapa de IET produzido por sensoriamento remoto.*

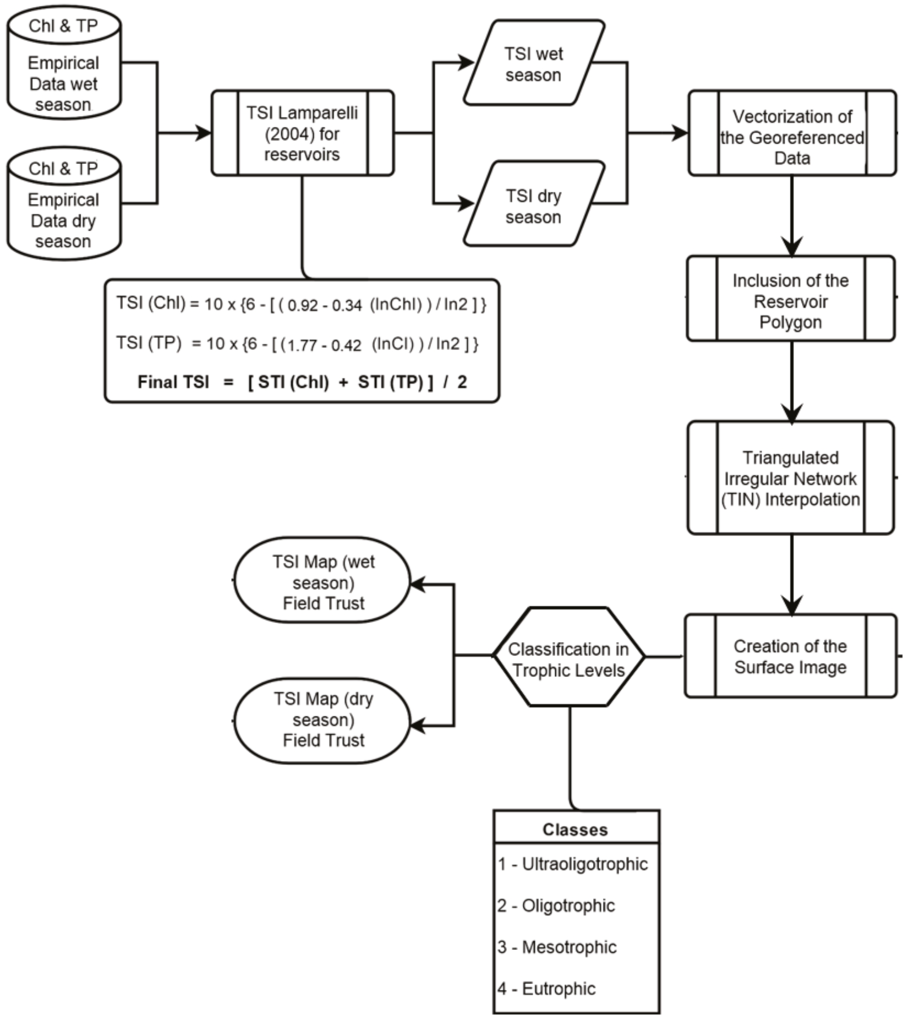

Figure 7. Flowchart showing the steps to perform the TSI Empirical Map. *Fluxograma apresentando as etapas de desenvolvimento do Mapa de IET Empírico.*

(see Table 1). The euphotic zone mask, that we create to constraint the area to perform the TSI RS, excluded 11.5 % of the pixels (51.5 ha) to avoid the bottom effects of shallow areas. We did not observed differences between the wet and dry season.

We create the TSI RS Maps and the Empirical TSI Maps as shown in Figure 6 and Figure 7, respectively. The resulting maps represents the TSI obtained by SR (Fig. 8) and the TSI obtained empirically, using the chlorophyll-α and Total P data (Fig. 9). Both maps comprise mainly the mesotrophic class in both periods. The dry season presents an increasing of values classified into the mesotrophic class, followed by a decrease of values located into the oligotrophic class, indicating an increase of trophic degree from wet to dry season. When we compared the TSI RS Maps (observed) with the Empirical TSI Maps (expected) we found no significant differences between the observed and the expected for both seasons (x^2 = 1, df = 27, with critical value (0.005) of 49.64). The Kappa Index also validate the TSI RS, since the Kappa Index was reasonable (0.2180) for the wet season and very good (0.6250) for the dry season.

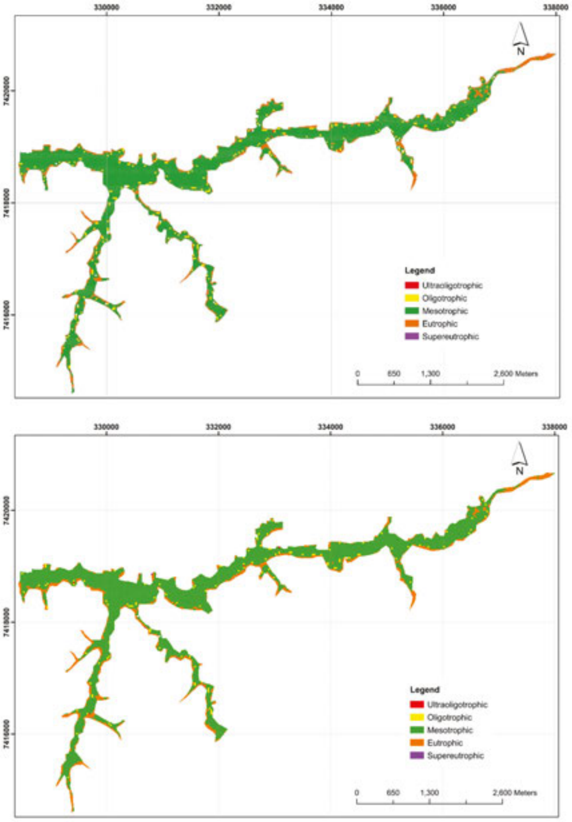

Figure 8. The TSI RS Map: a) dry season; b) wet season. *Mapa de IET produzido por sensoriamento remoto: a) estação seca; b) estação chuvosa.*

DISCUSSION

Our goal was to show ways to use orbital remote sensing data to determine the trophic state degree in an artificial reservoir, diminishing the costs of data acquisition and optimizing the monitoring of reservoirs. The bathymetric map was obtained using field survey and geoprocessing techniques. We also developed a series of flowcharts that presents the step by step to obtain all maps used here to model the Trophic State Index.

The bathymetric survey allows us to model the submerged relief, to delimit the reservoir polygon, and to create the euphotic zone mask by acquiring the X and Y coordinates, the Z elevation of the water surface, and the reservoir depth, within an adequate scale and accuracy to limnologic studies. The altimetry variation of 25 m, observed in the Relief Map is related to the preterit use of the reservoir area (sand mining), before the dam construction in the 1960's.

We obtained a homogeneous distribution of Secchi depth values along the reservoir in agreement with Pereira *et al.* (2011). They did not find relevant differences between the values obtained by RS of those obtained *in situ*, as we found in the present work. We expect to find some differences because the radiance within each pixel comprises the mean radiance of an area that varies with the spatial resolution of the satellite, whereas the field measurement is only one human observation. Therefore, the RS is much more reliable in obtaining the Secchi depth considering the limitations related to the spatial scale, whereas the Secchi depth is an inference and not a direct measure.

The use of euphotic zone mask is found to be necessary to avoid the undesirable bottom effect always present in RS data. The shallow areas must be erased because only the water within the euphotic zone can be related to limnologic parameters. Depending on the reservoir geomorphology the shallow waters can be significant or not. In this case they may not be significant, but we decide to maintain the mask in the flowchart to alert about its importance. The differences between the wet and dry season is perfectly understandable because the precipitation within the watershed during the wet season may strongly improve the turbidity. Ngabirano *et al.* (2016) also observed low turbidity during the dry season in reservoirs located in Uganda. They related the difference between both seasons as a decrease of concentration of small clay minerals particles, dissolved colored compounds and colloids in dry season.

However, it does not seem to be the case in our reservoir because it occurs in the plateau with Litolics rock outcrops, Cambisols, Yellow Red Latosol, and Podzolic Red Yellow soils. Analyzing Figure 2, it is possible to understand why the Paiva Castro reservoir is so peculiar: it is hilly, showing few shallow areas; it is the fifth reservoir of the Cantareira System, and it is kept in an almost constant water level along the seasons. The land used within its watershed (area of about 33 690 ha) comprises an urban areas (Mairiporã city) and some disperse human occupation (1.7 %), water of various type (1.6 %), cultivate forest (3.9 %), semi natural fields (33.4 %), and semi natural forest (59.4 %), according to Martins (2017) and Kumazawa *C* (2018).

The TSI RS Map produced here was geostatistical validated, whereas no significant differences were found between the observed (TSI RS) and the expected (Empirical TSI). Our results agree with Novo *et al.* (2013) about the TM4/TM3 ratio being appropriate to cover a large range of chlorophyll-*a* concentration and, consequently, to access a broad of trophic states degrees, favoring the identification of eutrophic areas in inland water systems with low turbidity. The TM4/TM3 ratio is the best input variable to estimate the chlorophyll-a concentration since the absorption peak occurs in 670 nm (RED) and the spectral reflectance peak (fluorescence and phytoplankton cell backscattering) occurs in 700 nm (NIR) besides this ratio mitigates the effects of the atmospheric and water surface reflectivity on the measurements performed by the satellite sensors (Novo *et al.*, 2013; Duan *et al.*, 2007).

CONCLUSION

We found that the bathymetric survey is necessary to model the submerged relief. We strongly advise the exclusion of the shallow areas before use satellite images (SR) to infer any limnologic parameters within the euphotic zone. We assumed that the Secchi depth obtained by SR tends to be more reliable because each pixel reveals the mean value of a bigger area than the observed in the field. The high concentration of chlorophyll-*a*, one of the symptoms of eutrophication, can be detected and measured by using satellite images due to the interaction between the pigment and the electromagnetic radiation. Thereby, it is possible to acquire a large amount of data, in several scales, that is useful to determine the trophic degrees in reservoirs. The strength of the RS relies on the easy access of information, in different space and time scales, and the acquisition of images can be without cost in some governmental agencies' website. Our results showed that RS can be reliable providing the information necessary to obtain the TSI and reinforces the potentiality of the RS to predict limnologic parameters in different space and time scales.

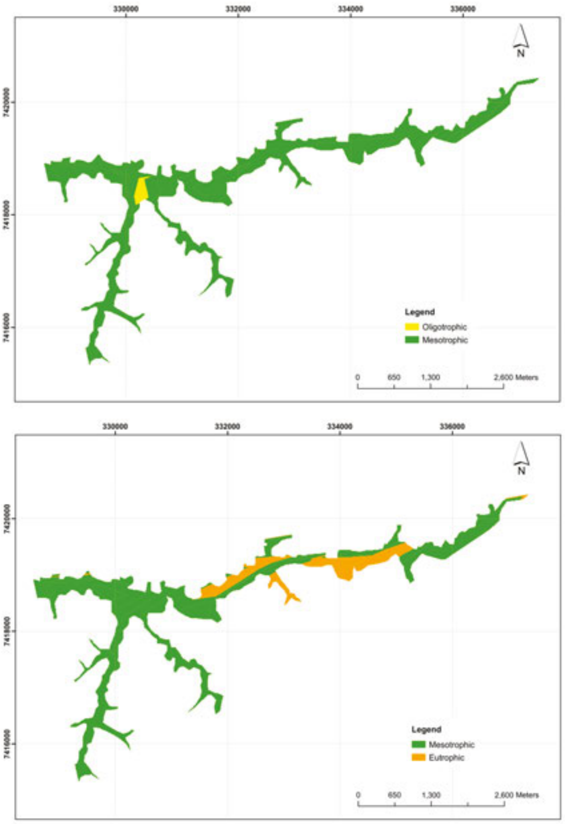

Figure 9. The TSI Empirical Map: a) dry season; b) wet season. *Mapa de IET Empírico: a) estação seca; b) estação chuvosa.*

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Calibration and validation of algorithms for the estimation of chlorophyll-*a* concentration and Secchi depth in inland waters with Sentinel-2

Marcela Pereira-Sandoval[1,*], Esther Patricia Urrego[1], Antonio Ruiz-Verdú[1], Carolina Tenjo[1], Jesús Delegido[1], Xavier Soria-Perpinyà[2], Eduardo Vicente[2], Juan Soria[2] and José Moreno[1]

[1] IPL - University of Valencia. Catedrático José Beltrán, 2. 46980 Paterna, Valencia (Spain).
[2] Institut Cavanilles de Biodiversitat i Biología Evolutiva (ICBiBE). Universitat de València. C/ Catedrático José Beltrán, 2. 46980-Paterna, València, (Spain).

[*] Corresponding author: Marcela.Pereira@uv.es

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ABSTRACT

Calibration and validation of algorithms for the estimation of chlorophyll-*a* concentration and Secchi depth in inland waters with Sentinel-2

Chlorophyll-*a* concentration and Secchi disk depth are two of the most important biophysical parameters used to assess water quality and determine the ecological state of inland waters. The *Ocean Color 2* and *Dall'Olmo three-band* algorithms were used to estimate chlorophyll-*a* concentration and the calibration of the ratio 490/705 nm was used to produce an algorithm for estimating Secchi disk depth. These algorithms have been calibrated for the Sentinel 2-Multispectral Instrument (S2-MSI) and validated using *in situ* measurements of chlorophyll-*a*, Secchi disk depth and radiometry. This data was taken in the Valencia region reservoirs as part of the project Ecological Status of Aquatic Systems with Sentinel Satellites (ESAQS). The results show that for estimating chlorophyll-*a* concentration, it is better to apply a prior classification based on their trophic status. For eutrophic and hypertrophic waters, the TBDO algorithm had an error of 23 mg/m^3 over a chlorophyll-*a* concentration range of between 10 to 169 mg/m^3. For ultraoligotrophic to mesotrophic waters, the better algorithm was OC2_490, which resulted in an error equal to 0.9 mg/m^3 over a chlorophyll-*a* concentration range of between 0.54 to 5.8 mg/m^3. For the estimation of water transparency by Secchi disk depth, we have obtained good results with the ratio 490/705 nm, with an error equal to 0.88 m over a Secchi disk depth range of between 0.26 to 8.1 m. These algorithms have been applied to S2-MSI images and satisfactory results have been obtained for different reservoirs in the Valencia region (Spain).

Key words: Ocean Color, Dall'Olmo three-band, chlorophyll-*a*, Secchi disk depth, Sentinel-2, HydroLight

RESUMEN

*Calibración y validación de algoritmos para la estimación de la concentración de la clorofila-*a *y profundidad de Secchi en aguas continentales con Sentinel-2*

*La concentración de clorofila-*a *y la profundidad del disco de Secchi son dos de los parámetros biofísicos más importantes utilizados para evaluar la calidad del agua y determinar el estado ecológico en aguas continentales. Los algoritmos Ocean Color 2 y triple banda de Dall'Olmo fueron aplicados para la estimación de la concentración de la clorofila-*a. *El ratio 490/705 nm se usó para producir un algoritmo para la estimación de la profundidad del disco de Secchi. Esos algoritmos han sido calibrados para el Instrumento Multiespectral de Sentinel 2 (S2-MSI) y validados usando medidas* in situ *de la clorofila-*a, *profundidad del disco de Secchi y radiometría. Estos datos se tomaron en los embalses de la región de Valencia en el contexto del proyecto Estado Ecológico de los Sistemas Acuáticos con Satélites Sentinel (ESAQS). Los resultados muestran que para la estimación de la concentración de la clorofila-*a *es mejor aplicar previamente una clasificación basada en su estado trófico. Para aguas eutróficas e hipertróficas, el algoritmo triple banda de Dall'Olmo tuvo un error de 23 mg/m*^{3} *sobre un rango de concentración de clorofila-*a *entre 10 a 169 mg/m*^{3}*. Para aguas ultraoligotróficas a mesotróficas el mejor algoritmo fue el*

*OC2_490, con el cual se obtuvo un error de 0.9 mg/m^3 sobre un rango de concentración de clorofila-*a *entre 0.54 a 5.8 mg/m^3. Para la estimación de la transparencia del agua mediante la profundidad del disco de Secchi, hemos obtenido buenos resultados con el ratio 490/705 nm, con un error igual a 0.88 m sobre un rango de profundidad de disco de Secchi entre 0.26 a 8.1 m. Estos algoritmos han sido aplicados a imágenes S2-MSI obteniendo resultados satisfactorios en diferentes embalses en la región de Valencia (España).*

Palabras clave: *Ocean Color, triple banda de Dall'Olmo, clorofila-*a*, profundidad de disco de Secchi, Sentinel-2, HydroLight*

INTRODUCTION

Chlorophyll-*a* concentration [Chl-*a*] is a phytoplankton biomass estimator in water bodies. It is one of the fundamental parameters of water quality used to detect algal blooms and assess eutrophication levels. The anomalous productivity of phytoplankton biomass relative to a "normal situation" (or the ecological optimum, depending on the watershed characteristics and climate of a given water body) is an indicator of eutrophication (Zheng and DiGiacomo, 2017). The [Chl-*a*] derivation from satellite data relies mostly on the absorption signal of phytoplankton. The variability of the [Chl-*a*] specific absorption coefficient is spectrally minimal around the red absorption peak (670 nm), where the influence of accessory pigments is minimal, and is higher around the blue absorption peak (440 nm), where the absorption of accessory pigments and dissolved organic matter is also high (Bricaud *et al.*, 1995; Stramsky *et al.*, 2001, Zheng & DiGiacomo, 2017).

Secchi disk depth (Z_{SD}) is a measurement of water transparency. The depth at which the disk disappears into the water is inversely proportional to the average amount of organic and inorganic materials along the path of sight in the water. The sense of sight is an integral part of the measurement procedure (Preisendorfer, 1986).

The European Water Framework Directive (WFD) establishes a framework for community-wide action for assessing the ecological status, management and conservation of the different water bodies in the member states. In this context, and in view of the need for comprehensive management of the aquatic systems of the Valencia region (south-eastern Spain), the Ecological Status of Aquatic Systems with Sentinel satellites (ESAQS) project was developed. The Regional Water Authority (Confederación Hidrográfica del Júcar, CHJ) collaborates and helps this project. The main objective of ESAQS is to develop and validate algorithms for the estimation of ecological quality indicators of inland waters (i.e. chlorophyll-*a* concentration, Secchi depth, colored dissolved organic matter and suspended solids) using the data provided by the Multispectral Instrument (MSI) sensor on-board the Sentinel-2 (S2-MSI) mission. The MSI scenes have a spatial resolution of 10 m, a high temporal resolution of 5 days and an adequate spectral resolution. Table 1 summarizes the principal features of S2-MSI. These characteristics make this sensor an exceptional instrument for the retrieval of biophysical parameters in inland waters, as compared to other satellite missions designed either for ocean color applications (e.g. the Ocean and Land Colour Imager, OLCI, on Sentinel-3 or the Moderate-Resolution Imaging Spectroradiometer, MODIS-Aqua), or the land-oriented Operational Land Imager (OLI) Landsat-8, which have a lower spatial resolution (300, 1000 and 30 m, respectively). S2-MSI data is suitable for the study of small irregularly-shaped water masses like most reservoirs in the region (Fig. 1) and small area as 80 ha (Regajo reservoir). Remote sensing data adds an extra value to limnological data collected from traditional *in situ* measurements (Giardino *et al.*, 2001), thanks to the synoptic view and frequency of the satellite passes. Remotely-sensed optical imagery over water bodies has been used as a cost-effective way for monitoring water quality.

The aim of this paper is to present the results of the calibration of water quality algorithms for the estimation of [Chl-*a*] and Z_{SD} from S2-MSI spectral bands and the validation with *in situ* [Chl-*a*] and Z_{SD} measurements. These algorithms cover a wide range of trophic states, from hypertrophic to ultraoligotrophic, in line with the characteristics

Table 1. Main characteristics of S2-MSI spectral bands. *Principales características de las bandas espectrales de S2-MSI.*

Band number	Central wavelength (nm)	Bandwidth (nm)	Spatial resolution (m)
1	443	20	60
2	490	65	10
3	560	35	10
4	665	30	10
5	705	15	20
6	740	15	20
7	783	20	20
8	842	115	10
8a	865	20	20
9	945	20	60
10	1380	30	60
11	1610	90	20
12	2190	180	20

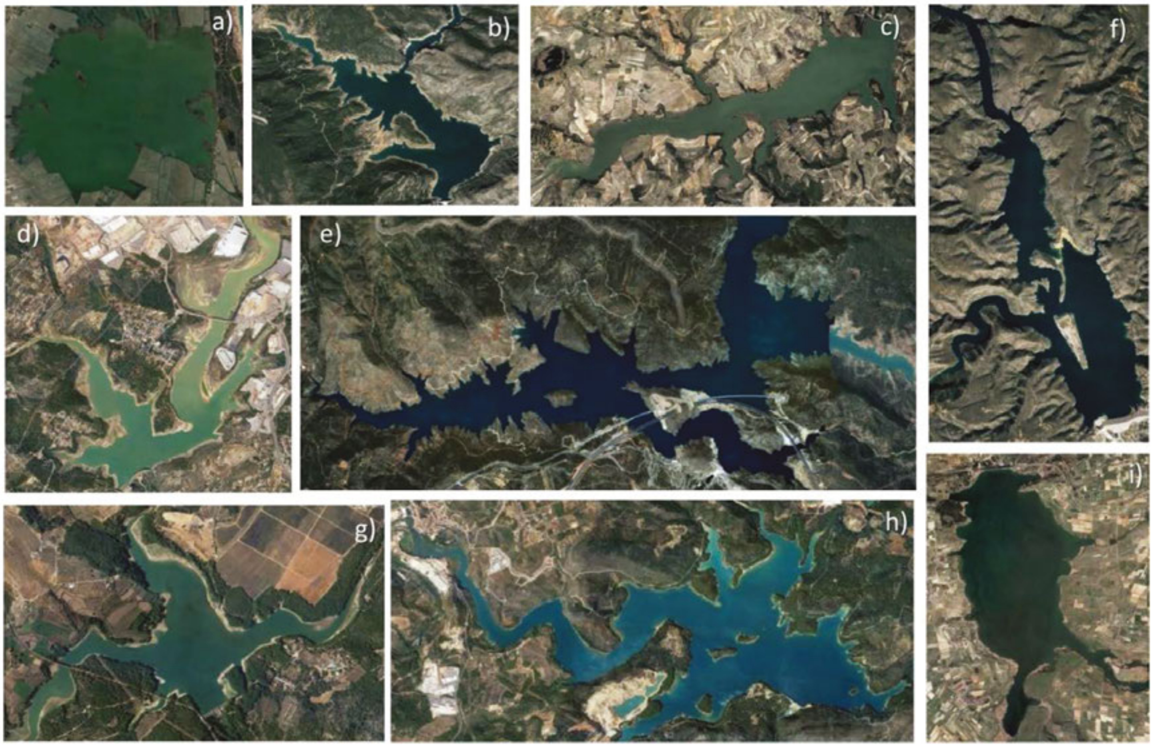

Figure 1. Reservoirs in study area. a) Albufera of Valencia, b) Benagéber, c) Beniarrés, d) María Cristina, e) Contreras, f) Tous, g) Regajo, h) Sitjar and i) Bellús. *Embalses en el área de estudio. a) Albufera de Valencia, b) Benagéber, c) Beniarrés, d) María Cristina, e) Contreras, f) Tous, g) Regajo, h) Sitjar y i) Bellús.*

of reservoirs measured in our study region. This objective fits into the main ESAQS goal of formulating new algorithms for inland water ecological state monitoring from remote sensing.

DATA AND METHODS

Study area

Since 2017, the first year of the ESAQS project, a large number of field campaigns have been carried out in the Valencia region. The study area includes the reservoirs of Benagéber, Bellús, Beniarrés, Contreras, María Cristina, Regajo, Sitjar, Tous and the Albufera of Valencia (Fig. 2), covering a wide gradient of trophic states. For each reservoir, between 1 and 4 *in situ* measuring points were taken at a suitable distance from shoreline to avoid mixed pixels (land-water mixed reflectance). The field campaigns were planned on cloud-free days on which the S2 satellites acquired images over the reservoir region.

In situ data: [Chl-*a*] and Z_{SD}

[Chl-*a*] and Z_{SD} were measured by the Limnology Research Team of the University of Valencia. [Chl-*a*] data were obtained from the water samples by the spectrophotometric method. Samples were filtered through 0.4-0.6 µm GF/F glass

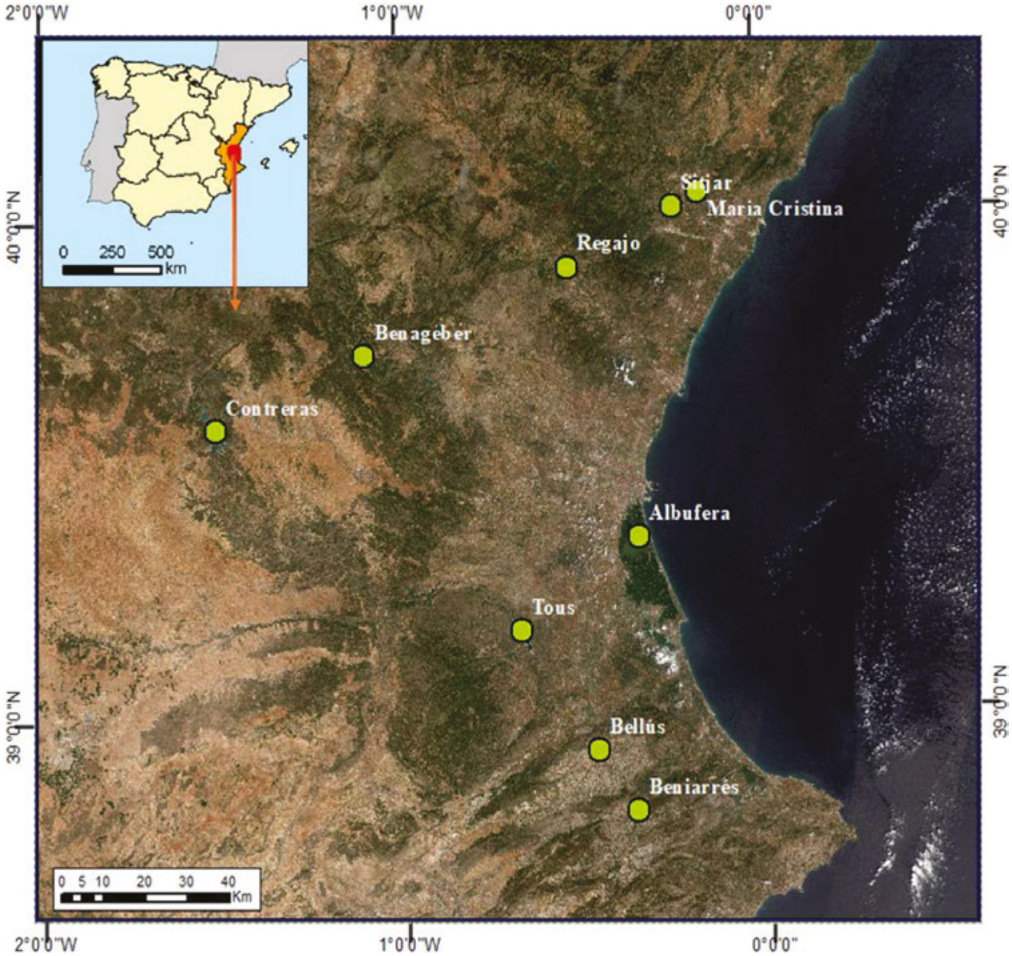

Figure 2. Location map of reservoirs and lakes in study in Valencia region. *Mapa de localización de los embalses y lagos en estudio en la región de Valencia.*

fiber filters, extracted according to standard methods (Shoaf & Lium, 1976) and the calculation methods by Jeffrey & Humphrey (1975) (Soria *et al.*, 2019). The range of *in situ* [Chl-*a*] measurements in the study area reservoirs was between 0.54 to 169 mg/m^3.

Z_{SD} data was measured with a metal disk called a Secchi disk. It is divided into four quarters, each one painted black and white. The measurement procedure for obtaining the Z_{SD} is to slowly lower it into the water until it disappears from sight. When that happens, it is possible to obtain the Z_{SD}. The *in situ* Z_{SD} measurement range in the reservoirs under study was between 0.25 to 10.5 m.

In situ reflectances

The *in situ* reflectances were calculated from above-water *in situ* measurements of: 1) The water-leaving radiance (L_w), 2) the sky radiance (L_{sky}) and 3) the downwelling solar irradiance (E_S). These were measured using a HandHeld2 radiometer, which has a wavelength range of 325 to 1075 nm and a spectral resolution of 1 nm; and an Ocean Optics (HR 4000) radiometer ranging from 200 to 1100 nm at a spectral resolution of ~0.2 nm. According to previous studies (Mobley, 1999) and recommendations done by international remote sensing ocean groups (Fargion & Mueller, 2000), the measurements have been taken with a view zenith angle of ~45 º and an azimuthal angle of ~135 º, with respect to the sun, to minimize the direct and diffuse sunlight reflected by the water surface (Mobley, 1999). Once the measurements were obtained, the *in situ* reflectance spectrum was convolved to the S2-MSI spectral bands (Table 1). This procedure was done to fit the *in situ* reflectance spectral resolution (~0.2 nm or 1 nm) to S2-MSI bandwidth using the Spectral Response Functions database (SRF v2.0) (ESA, 2018a). The SRF determine the position and width of each S2-MSI spectral band (D'Odorico *et al.*, 2013).

S2-MSI imagery

The Cloudless Level 1C S2-MSI images were downloaded from the Copernicus Open Access Hub of the European Spatial Agency (ESA, 2018b) to be concurrent with the *in situ* measurements. SNAP software v.5.0 (ESA, 2018c) was used for the image processing. For the specific case of the Albufera of Valencia, images were atmospherically corrected to Bottom of Atmosphere (BOA) reflectances using the Sen2Cor processor (ESA, 2018d). While this atmospheric correction method was designed for land products, it gives back optimum results in eutrophic waters like the Albufera of Valencia according to Ruescas *et al.* (2016) and Soria *et al.* (2017a). In this respect, we would highlight the importance of atmospheric correction over inland waters. In the ESAQS project, we are evaluating different atmospheric correction methods to obtain an optimum retrieval of the algorithms calibrated and validated to actual S2-MSI images for routine monitoring purposes.

Calibration and validation of established [Chl-*a*] algorithms

To calibrate the [Chl-*a*] algorithms and encompass the variability of the optically active components present in inland waters, we generated a calibration database of simulated data (N = 392) by using the radiative transfer model HydroLight (Mobley, 1994). We carried out simulations with a three-component Case-2 model with ranges of variables characteristics for oligotrophic to eutrophic waters: [Chl-*a*] (1-500 mg/m^3), Colored Dissolved Organic Matter (CDOM) (0.01-4 m^{-1}) and Non-Algal Particles (NAP) (0.1-100 g/m^3). To estimate [Chl-*a*] with S2-MSI spectral bands, we tested the Ocean Color 2_443 nm (OC2_443) and Ocean Color 2_490 nm (OC2_490) (replacing the original 483 band with the 490 band in S2-MSI), the Ocean Color 3 (OC3) (O'Reilly *et al.*, 2000) and the Dall'Olmo three-band algorithm (Dall'Olmo *et al.*, 2003), hereafter called the TBDO algorithm. Following the methodology of Ruiz-Verdú *et al.* (2016), the OC2 and OC3 algorithms, based on the ratio of green and blue bands, have been recalibrated for the S2-MSI spectral bands. The TBDO algorithm, which is based on the evaluation of the reflectance at red and near infrared bands (665, 705 and 740 nm), is used for the estimation of [Chl-*a*] in inland and coastal water (Gilerson *et al.*, 2010). This algorithm has also been calibrated for the S2-MSI spectral bands and validated in a previous work in a hypertrophic coastal lagoon: the Albufera of Valencia (Guibaja *et al.*, 2016).

The [Chl-*a*] algorithms used in this work are described below. The general equation for the OC2 and OC3 models are:

$$\log_{10}[\text{Chl-}a] = a + bX + cX^2 + dX^3 \quad (1)$$

where X is calculated according to:

OC2_443 model (2)

$$X=\log_{10}[Rrs\_443/Rrs\_560]$$

OC2_490 model (3)

$$X=\log_{10}[Rrs\_490/Rrs\_560]$$

OC3 model (4)

$$X=\log_{10}[\max(Rrs\_443; Rrs\_490)/Rrs\_560]$$

The Rrs_443, Rrs_490 and Rrs_560 are reflectances of S2-MSI spectral bands.

The algorithm called OC2_490, fit to the S2-MSI bands, is set to the spectral band 490.

The three-band model of Dall'Olmo *et al.* (2003) (TBDO), takes the form:

$$[\text{Chl-}a] = aX^2 + bX + c \quad (5)$$

where

$$X= [Rrs\_740*((Rrs\_665)^{-1} – (Rrs\_705)^{-1})] \quad (6)$$

The Rrs_665, Rrs_705 and Rrs_740 are reflectances of S2-MSI spectral bands.

Once the different algorithms were calibrated from the HydroLight database, we validated it with the [Chl-*a*] field data, selecting the best method according to different statistical parameters. If a linear trend is maintained, but away from the 1:1 line, the algorithms were recalibrated.

Calibration and validation of Z_{SD} algorithms

The attenuation of light into water bodies and the vertical visibility (water transparency) is described by the Secchi disk depth (Z_{SD}). It is linked to two optical parameters: the vertical diffuse attenuation coefficient K_d (m^{-1}) and the attenuation coefficient c (m^{-1}) (Antoine, 2010). The vertical visibility is analogous to Z_{SD} and it is the inverse of the sum K_d + c (Doron *et al.*, 2007). Austin and Petzold, (1981) estimated K_d (490 nm) from blue-green water leaving radiances. This algorithm was modified by Kratzer *et al.*, (2008) to estimate Z_{SD} for remote sensing applications (Alikas & Kratzer, 2017). The ratio 490/560 is used to map transparency over clear waters, typically in open ocean waters (Mueller, 2000; Giardino *et al.*, 2001; Antoine, 2010; Soria *et al.*, 2017b). This ratio is a good estimator of Z_{SD} in clear waters in which the phytoplankton is the main contributor to the light attenuation in the water column.

To estimate the transparency of the water in this work, we have performed a process of re-calibration of the 490/560, and 490/705 ratios, plus the hereafter called "Koponen" method (Koponen *et al.*, 2001) after testing different algorithms. We have modified the original Alikas & Kratzer (2017) algorithm. In this paper, the band ratio are not raised to the exponent b. Instead, the b coefficient is added or subtracted depending on the direction of the slope, obtained in the relation between *in situ* Secchi disk depth (Z_{SD}) measurements and band ratio. The algorithm is detailed in equation 7:

$$Z_{SD} = e\^{} (a*\ln[Rrs\_490/Rrs\_560] + b) \quad (7)$$

where

Rrs_490 and Rrs_560 are reflectances of S2-MSI spectral bands at 490 and 560 nm.

a and b are the coefficients derived by linear regression between the Napierian logarithm of *in situ* Z_{SD} and the Napierian logarithm of 490/560 ratio.

Alikas & Kratzer (2017) applied another model based on ratio 490/705. This model had a better result (R^2 = 0.73) in the Himmerfjärden lake with a 1.9 to 7.5 m Z_{SD} and a 1.2 to 11.6 mg/m^3 [Chl-*a*] range (Baltic Sea). The algorithm is detailed in equation 8:

Z_{SD} = e^ (a*ln [Rrs_490/Rrs_705] + b) (8)

where

Rrs_490 and Rrs_705 are reflectances of S2-MSI spectral bands at 490 and 705 nm.

a and b are the coefficients derived by linear regression between Napierian logarithm of *in situ* Z_{SD} and the Napierian logarithm of 490/705.

In more turbid waters, where other optically active constituents such as NAP and CDOM are also present in significant concentrations, other spectral regions are more sensitive for estimating the Z_{SD} (Doron *et al.*, 2007). Since this was the present situation in some reservoirs studied in ESAQS, other algorithms were also tested, such as the Koponen *et al.*, (2001) algorithm. This algorithm was validated in Finnish Lakes where the water bodies generally present Z_{SD} values of less than 3 m. We have modified the original algorithm since we have not applied the 783 band. The modified Koponen algorithm is detailed in equation 9:

Z_{SD} = e^ (a*ln [Rrs_560/Rrs_705] + b) (9)

where

a and b are the coefficients derived by linear regression between Napierian logarithm of *in situ* Z_{SD} and the Napierian logarithm of 560/705.

The Rrs_560 and Rrs_705 are reflectances of S2-MSI spectral bands.

RESULTS

The *C* Z_{SD} measurement range in the water bodies under study was between 0.25 to 10.5 m. The range of Limnetica, 38(1): 467-479 (2019). DOI: 10.23818/limn.38.26 [Chl-*a*] measurements in the study area water bodies was between 0.54 to 169 mg/m^3.

Calibration and validation of [Chl-*a*] algorithms

In the set of simulated data used for calibration, the input [Chl-*a*] presented a very high correlation, according the coefficient of determination (R^2), with the retrieved [Chl-*a*] in all algorithms: OC2_443 with a R^2 = 0.98 (Fig. 3a); OC2_490 with R^2 = 0.97; (Fig. 3b); OC_3 with R^2 = 0.97 (Fig. 3c); and TBDO with R^2 = 0.99 (Fig. 3d). The specific calibration coefficients of each algorithm were obtained by a polynomial fit between the simulated [Chl-*a*] database and reflectance band ratios. Table 2 summarizes the coefficients of each polynomial fit.

To validate these algorithms, the data measured in the field was used. Table 3 shown this dataset. According to Guibaja *et al.* (2015), the TBDO algorithm had an optimum performance on eutrophic and hypertrophic waters. In our study area, this type of water corresponds to reservoirs with [Chl-*a*] > 10 mg/m^3 like the Albufera of Valencia, and the Bellús and Beniarrés reservoirs (Table 3). For [Chl-*a*] < 10 mg/m^3, we have applied the OC2_443, OC2_490 and OC3 algorithm on ultraoligotrophic, oligotrophic and mesotrophic waters. These water types correspond to the Benageber, Tous, María Cristina, Contreras, Sitjar and Regajo reservoirs (Table 3). The error analysis was performed between the calibrated algorithms and *in situ* [Chl-*a*] measurements. For this analysis, we have applied the mean absolute error (MAE) and a systematic direction of the error (bias). Figure 4 shows the [Chl-*a*] measured *in situ* versus the calculated with the 4 algorithms. In the OC2_443, OC2_490 and OC3 algorithms, it can be seen that the slope presented values very far from line 1:1 (0.25 and 0.30) (Fig. 4a, b and c) with a very high bias. This means that in these cases, ocean color models are overestimating the actual chlorophyll concentration, with high error values. For this reason, it was necessary to perform a recalibration process to readjust each of the models. Table 4 shows the final equation, which was obtained from the recalibration process for the [Chl-*a*] estimation, replacing the x of each adjustment (Fig. 4 a, b and c) the formulas obtained in the calibration process. For this, the value of the line slope (coefficient a) was integrated logarithmically in equation 1.

According to Table 4 data, all three algorithms provide similar statistics, but the OC3 was the algorithm which gave back the lowest error equal

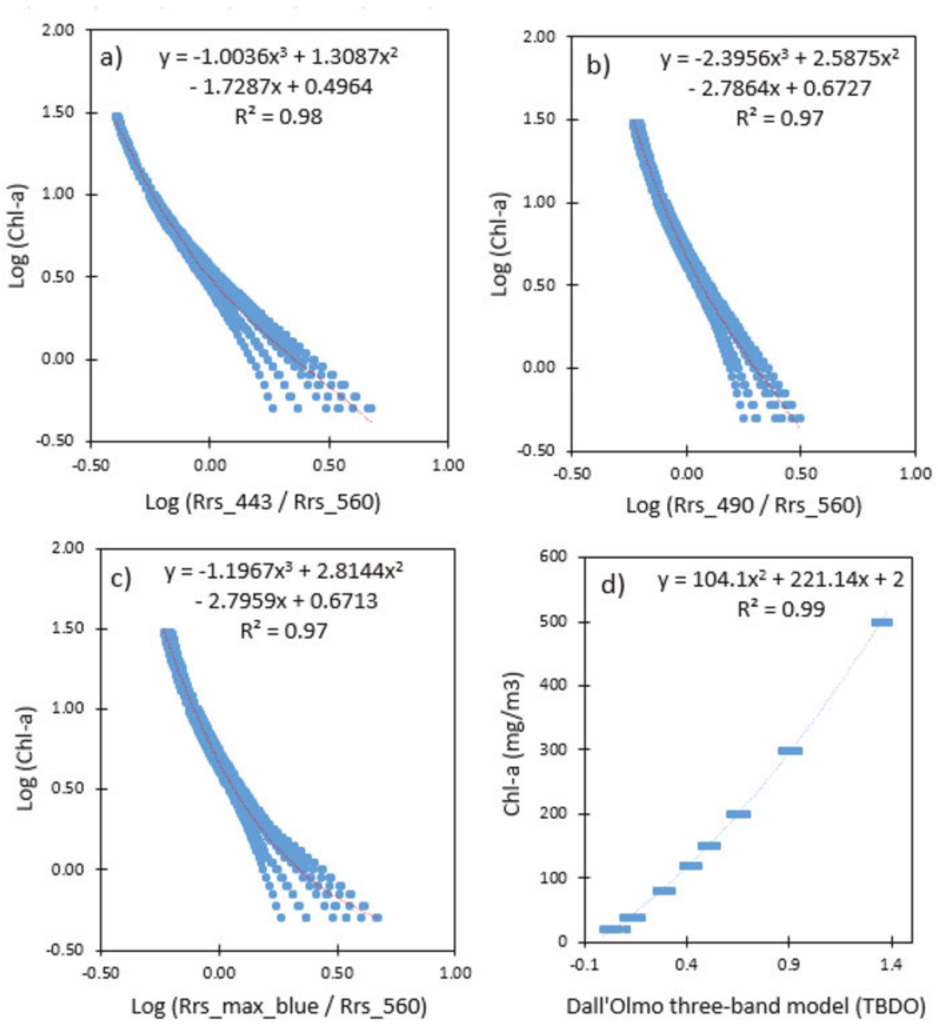

Figure 3. a) OC2_443 algorithm and simulated [Chl-*a*] polynomial fit. b) OC2_490 algorithm and simulated [Chl-*a*] polynomial fit. c) OC3 algorithm and simulated [Chl-*a*] polynomial fit, and d) TBDO algorithm and simulated [Chl-*a*] polynomial fit. *a) Ajuste polinómico entre el algoritmo OC2_443 y la [Chl-*a*] simulada. b) Ajuste polinómico entre el algoritmo OC2_490 y la [Chl-*a*] simulada. c) Ajuste polinómico entre el algoritmo OC3 y la [Chl-*a*] simulada, y d) Ajuste polinómico entre el algoritmo TBDO y la [Chl-*a*] simulada.*

Table 2. Coefficients obtained by polynomial fit for OC2_443, OC2_490, OC3 and TBDO algorithms for [Chl-*a*] estimation (in mg/m^3) from simulated HydroLight dataset. *Coeficientes obtenidos por ajuste polinomial para los algoritmos OC2_443, OC2_490, OC3 y TBDO para la estimacion de la [Chl-*a*] (en mg/m^3) a partir de una base de datos simulada con HydroLight.*

Algorithm	**a**	**b**	**c**	**d**
OC2_443	0.4964	-1.7287	1.3087	-1.0036
OC2_490	0.6727	-2.7864	2.5875	-2.3956
OC3	0.6713	-2.7959	2.8144	-1.1967
TBDO	104.1	221.1	2.0	

Table 3. Database of *in situ* [Chl-*a*] and Secchi depth disk measurements in Valencia region reservoirs and lakes according by field campaign date. *Base de datos de medidas* in situ *de [Chl-*a*] y profundidad del disco de Secchi en los embalses y lagos de la región de Valencia según fecha de campaña de campo.*

Reservoir	[Chl-*a*] (mg/m³)	Z_{SD} (m)	Date
Albufera	51.3, 53, 54.2, 31.8, 39.1, 43.1		05/08/2015
Albufera	54.8, 56.3, 56.1,52.9,55.2, 58.3		27/08/2015
Albufera	148.9, 157.9, 156, 169.1, 159.4, 154, 93.3		30/11/2015
Albufera	135.6, 138.2, 121.5, 25, 128.7	0.31, 0.31, 0.33, 0.43, 0.32	12/03/2016
Albufera	141.8, 130.4, 140.1, 114.6, 78.1, 84.4, 83.2	0.25, 0.26, 0.3, 0.35, 0.26, 0.33	21/04/2016
Albufera	70.4, 68, 22.4, 10.7, 36.4, 43.5	0.3, 0.32, 0.33, 0.34,0.34, 0.37, 0.5	02/05/2016
Tous	1.27, 1.78, 3.1	5.9, 6, 5.8	27/12/2016
Bellús	31.8	1	16/01/2017
Contreras	2, 1, 0.79, 0.83	1, 1.3, 1.07	08/02/2017
Albufera	39.7, 64.5, 45.1, 47.3	0.27, 0.32, 0.34, 0.36	07/03/2017
Beniarrés	45.45	0.95	27/03/2017
Benagéber	2.48, 2.74	4, 5.2, 7.4	30/03/2017
Ma. Cristina	1.4, 1.33	5.2, 5.6	06/04/2017
Sitjar	0.54, 0.65	9.4, 10.5	06/04/2017
Bellús	61.39, 66.76, 68.01	0.55	15/06/2017
Regajo	8.6, 8.9, 10.2	2, 1.7	05/07/2017
Sitjar	0.68, 0.61	2.7, 3.15	23/10/2017
Benageber	5.4, 5.76, 4.57	3.4, 3.6, 4.1	26/10/2017
Beniarrés	11.13, 17.17	1.15, 1.4	07/11/2017
Tous	0.64, 0.72, 0.69	7.1, 8, 9.1	17/11/2017
Contreras	2.08, 2.42, 2.02, 0.86, 0.98	4.1, 5	30/11/2017
Tous	0.63, 0.58, 0.69	7, 7.75, 8.1	16/01/2018
Ma. Cristina	2.92, 2.72	0.75, 0.75	31/01/2018
Sitjar	0.59, 0.63	2.4, 2.2	31/01/2018
Benageber		4.3, 4.85, 5.5	23/02/2018
Albufera	84.5, 82.1, 81.6	0.31, 0.33, 0.30	07/03/2018
Bellús	41.54, 51.59, 49.09	0.45, 0.45, 0.5	22/03/2018
Regajo	5.57, 4.58, 4.63, 5.12	3, 3.75, 4, 4.25	11/05/2018
Benageber	4.91, 4.91, 4.85, 4.53	3.35, 3.35, 3.7, 3.75	16/05/2018

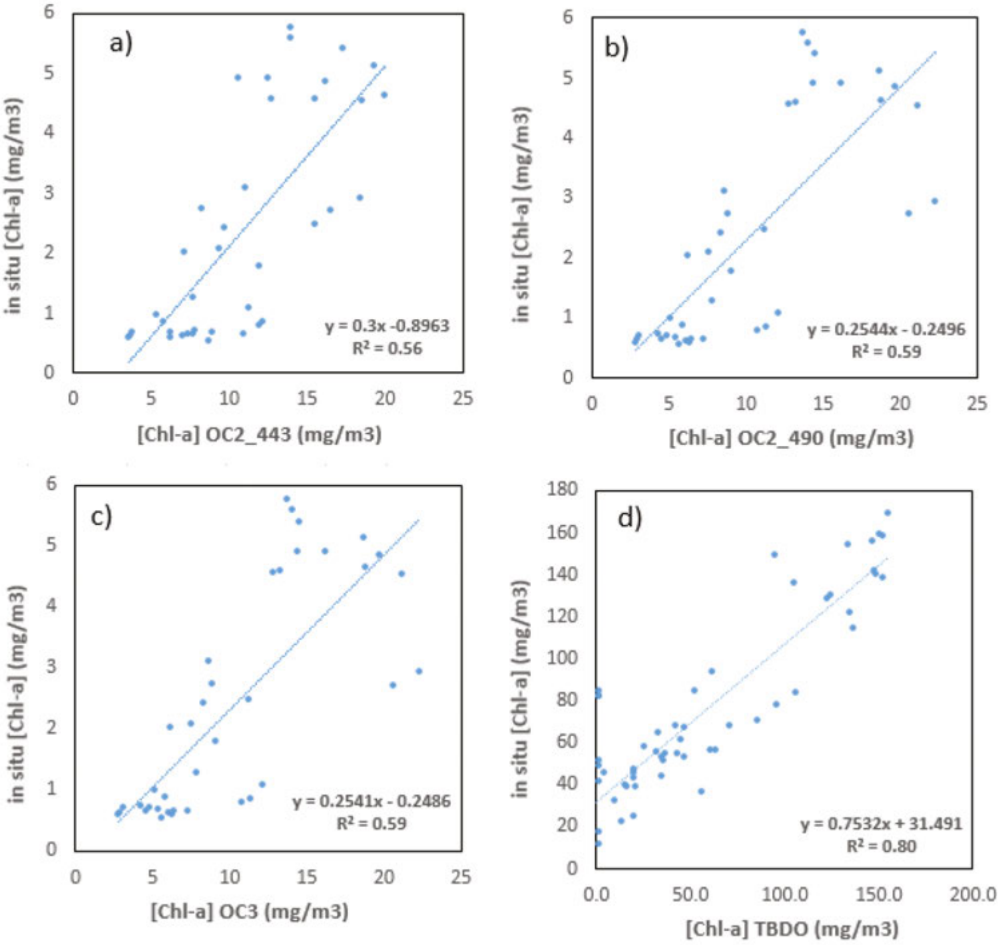

Figure 4. a) OC2_443 algorithm and *in situ* [Chl-*a*] validation. b) OC2_490 algorithm and *in situ* [Chl-*a*] validation. c) OC3 algorithm and *in situ* [Chl-*a*] validation, and d) TBDO algorithm and *in situ* [Chl-*a*] validation. *a) Validación del algoritmo OC2_443 en relación a la [Chl-*a*]* in situ. *b) Validación del algoritmo OC2_490 en relación a la [Chl-*a*]* in situ. *c) Validación del algoritmo OC3 en relación a la [Chl-*a*]* in situ*, and d) Validación del algoritmo TBDO en relación a la [Chl-*a*]* in situ.

to 0.89 mg/m^3. However, the OC2_490 model also has very good results, with the same correlation as OC3 and the advantage that it is easier to calculate, saving processing time and using only one blue band, versus OC3 that he need to use both.

For eutrophic waters with [Chl-*a*] > 10 mg/m^3, the TBDO algorithm (Fig. 4d) gave an $R^2 = 0.80$, a bias = 17.49 and an MAE = 23 mg/m^3, which is a relative low error for high [Chl-*a*] waters, taking into consideration the range of values for this group (10 to 169 mg/m^3). We consider it acceptable for the evaluation of the ecological status in these eutrophic or hypertrophic reservoirs (Guibaja *et al.*, 2016). The validated TBDO algorithm was applied to an S2-MSI image from the Albufera of Valencia acquired on 07/03/2018. This S2-MSI image was atmospherically corrected with Sen2-Cor. Fig. 5 shows the patterns of [Chl-*a*] concentration with medium to high values in nearly the entire lake with a [Chl-*a*] TBDO, between 75 and 90 mg/m^3. The same day, the *in situ* [Chl-*a*] measurements recorded in the Albufera of Valencia were between 81.6 to 84.5 mg/m^3 (Table 3). The green areas correspond to the water contributions with less chlorophyll-*a* coming from the irrigation channels renovating the water, while the central area corresponds to stagnant water, which leads to higher values of [Chl-*a*].

Table 4. Equations for the [Chl-*a*] estimation (in mg/m^3) in the calibration process. *Ecuaciones para la estimación de la [Chl-*a*] (en mg/m^3) en el proceso de calibración.*

Algorithm	Equation ([Chl-*a*] in mg/m^3)	MAE	R^2
OC2_443	[Chl-*a*] = 10^(-0.02648-1.7287*X+1.3087*X^2-1.0036*X^3)-0.8963	0.93	0.56
OC2_490	[Chl-*a*] = 10^(0.078217-2.7864*X+2.5875*X^2-2.3956*X^3)-0.2496	0.90	0.59
OC3	[Chl-*a*] = 10^(0.076305-2.7959*X+2.8144*X^2-1.1967X^3)-0.2486	0.89	0.59

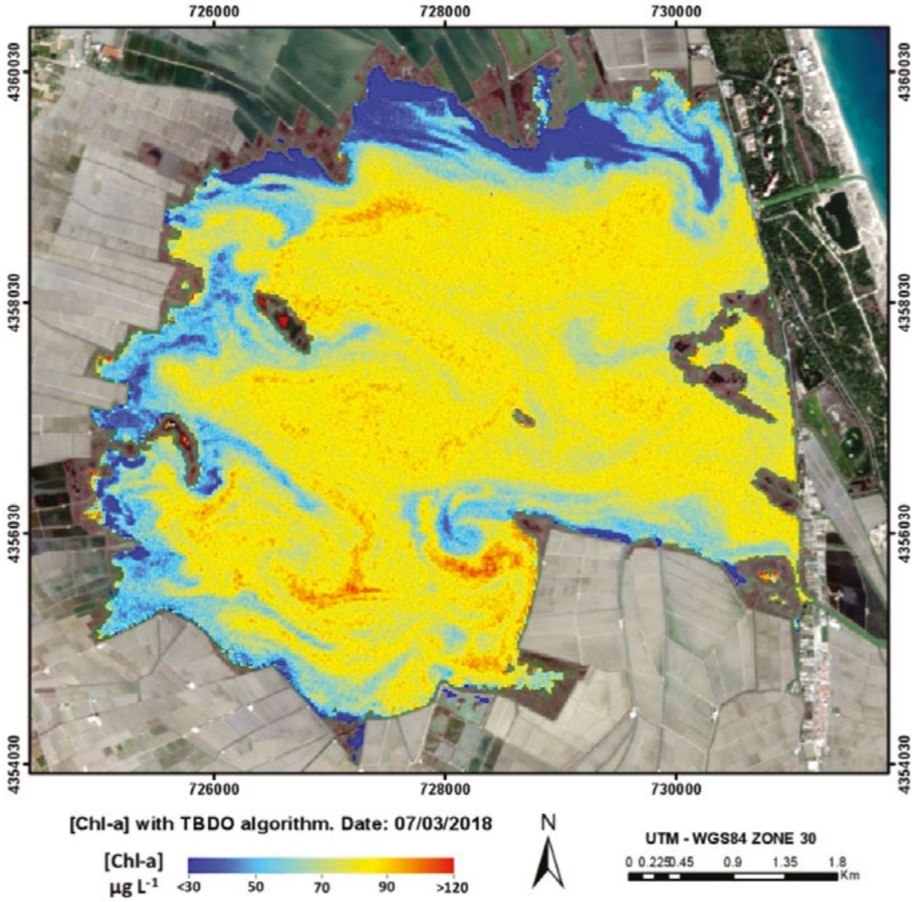

Figure 5. [Chl-a] map by TBDO algorithm in the Albufera of Valencia from S2-MSI image of 07/03/2018. *Mapa de [Chl-a] aplicando el algoritmo TBDO en la Albufera de Valencia obtenido de la imagen S2-MSI del 07/03/2018.*

Calibration and validation of Z_{SD} algorithms

The calibration-validation process of algorithms for the Z_{SD} estimation, has been based on the relationship between the 490/560, 490/705 and 560/705 ratios and *in situ* Z_{SD} measurements. Inspired by the algorithms described earlier, we have tested several regression models. The dataset collected in field campaigns over the study area consist of 79 measurements. It has a wide Z_{SD}

range from 0.25 to 10 m. For the calibration and validation process, the dataset was divided into 2 groups: 75 % of the data (N = 60) was used to calibrate and 25 % (N = 19) to validate the ratios. In a first attempt, we tested the three algorithms applying the trophic classification followed in the [Chl-*a*] algorithm calibration, differentiating ultraoligotrophic-mesotrophic waters and eutrophic-hypertophic waters, but the results were not optimum due to the limited dataset used.

Figure 6 shows the results of the calibration process. Here, the logarithm of measured Z_{SD} is represented, depending on the logarithm of each of the three indices. In Fig. 6 it is observed that the best fit is the linear one, and that for the calibration process, the 490/705 and 560/705 ratios showed the best performance in both cases.

To validate the Z_{SD} algorithms, we applied the coefficients obtained in the calibration process to the different band ratios, as explained earlier. The validation data set consists of 19 measurements (25 % of total). Figure 7 show the results. To select the best method, Table 5 shows the three formulas obtained in the calibration process and the statistics of the validation process. Considering all the statistics, the algorithm with the lowest error and the best correlation is 490/705.

According to these results, the 490/705 ratio is the most appropriate for estimating Z_{SD} with an error MAE of 0.88 m. We have applied this algorithm on the same S2-MSI scene that was used in the application of the [Chl-*a*] algorithm. We obtained a map that shows the different values of the water transparency of this hypertrophic lake (Fig. 8). For the Albufera of Valencia (Fig. 5 and Fig. 8), it can be observed that according to the 490/705 ratio, a vast majority of the lagoon had values between 0.30 to 0.36 meters. Once again, the correct match-up with the *in situ* Z_{SD} was confirmed. According to the data measured this day, the Z_{SD} was between 0.30 and 0.33 m (Table 3).

As can be seen in the [Chl-*a*] and the Z_{SD} maps, the expected relationship between the minimum value of chlorophyll concentration and maximum water transparency is given in areas near shoreline. In the northern region, the concentration of chlorophyll is minimum, taking into account the values normally measured in Albufera of Valencia (See Table 3). Here the values are between 50 to 70 mg/m^3, coinciding with maximum transparency, Z_{SD} up to 0.38 meters, again matching-up with the values registered in this lagoon (Table 3). This high transparency is probably due to the early opening of water gates for rice crops that usually starts in May.

DISCUSSION

To estimate [Ch-*a*] and according to the error analysis (MAE = 0.89 mg/m^3), the OC2_490

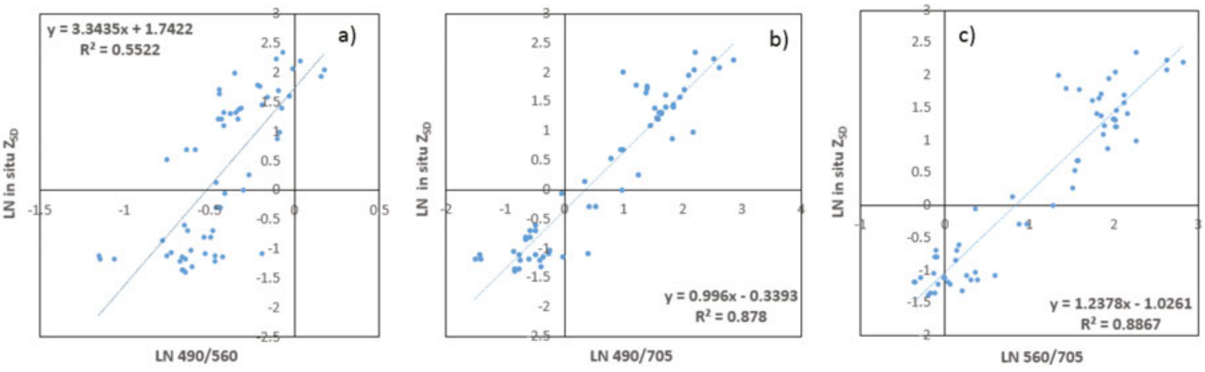

Figure 6. Calibration of the Z_{SD} algorithms. a) 490/560 algorithm and *in situ* ln(Z_{SD}) linear fit. b) 490/705 algorithm and *in situ* ln(Z_{SD}) linear fit. and c) 560/705 algorithm and *in situ* ln(Z_{SD}) linear fit. *Calibración de los algorimos para Z_{SD}. a) Ajuste lineal entre el algoritmo 490/560 y ln(Z_{SD})* in situ. *b) Ajuste lineal entre el algoritmo 490/705 y ln(Z_{SD})* in situ, *y c) Ajuste lineal entre el algoritmo 560/705 y ln(Z_{SD})* in situ.

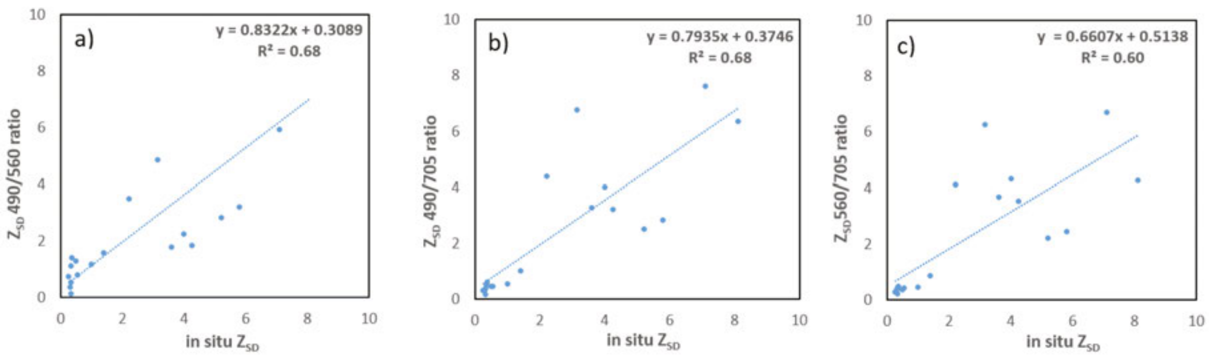

Figure 7. Validation of the Z_{SD} algorithms. a) Z_{SD} calculated with the 490/560 ratio in relation with *in situ* Z_{SD}. b) Z_{SD} calculated with the 490/705 ratio in relation with *in situ* Z_{SD} validation. c) Z_{SD} calculated with the 560/705 ratio in relation with *in situ* Z_{SD}. *Validación de los algorimos para Z_{SD}. a) Z_{SD} calculada con el ratio 490/560 en relación a Z_{SD} medida* in situ. *b) Z_{SD} calculada con el ratio 490/705 en relación a Z_{SD} medida* in situ. *c) Z_{SD} calculada con el ratio 560/705 en relación a Z_{SD}* in situ.

Table 5. Equations for the Z_{SD} estimation and statistics of the validation process. *Ecuaciones para la estimación de Z_{SD} y estadísticos del proceso de validación.*

Algorithm	**Equation** (Z_{SD} in m)	**R^2**	**MAE**	**Bias**
490/560	Z_{SD} = e^(3.3435*ln (Rrs_490/Rrs_560) + 1.7422)	0.68	1.16	0.12
490/705	Z_{SD} = e^(0.996*ln (Rrs_490/Rrs_705) - 0.3393)	0.68	0.88	0.15
560/705	Z_{SD} = e^(1.2378*ln (Rrs_560/Rrs_705) -1.0261)	0.60	0.96	0.35

algorithm should be preferred for low values ([Chl-*a*] = < 10 mg/m^3), as is the case of the reservoirs of Benagéber, Contreras, Sitjar, Tous, Beniarrés, María Cristina and Regajo. While Ocean Color algorithms have a longer tradition in ocean studies, the optimum results achieved in this study suggest they could have broad applicability on some types of inland waters. The validation of the TBDO algorithm gave a good result in eutrophic to hypertrophic water mass with [Chl-*a*] > 10 mg/m^3, such as the Albufera of Valencia and the Bellús reservoir. The results obtained in this study ratify the ones obtained by Guibaja *et al.* (2016) on the applicability of this algorithm on eutrophic to hypertrophic water masses. The match-up obtained between the TBDO application over the S2-MSI image and the values measured *in situ* the same day confirms the validity of the TBDO algorithm in water masses with high value of chlorophyll-*a*.

To estimate the Z_{SD}, we have obtained an optimum result with the 490/705 band ratio. While it is true that the environment and natural conditions between the Baltic Sea and the reservoirs of the Valencia region are different, the Z_{SD} range (0.25 to 10.5 m) measured in our study area is similar to that measured by Alikas & Kratzer (2017) in Himmerfjärden Lake (1.9 to 7.5 m). As happened with the [Chl-*a*], the match-up obtained between the 490/705 ratio application over the S2-MSI image and the values measured *in situ* the same day confirms the validity of this algorithm in water masses with lower water transparency value.

Regarding the applicability of the three algorithms by trophic states as we did for the [Chl-*a*] algorithms, we concluded that is not appropriate due to a) the limited dataset for the calibration-validation process and b) the composition of suspended solids of water, due to similar Z_{SD}

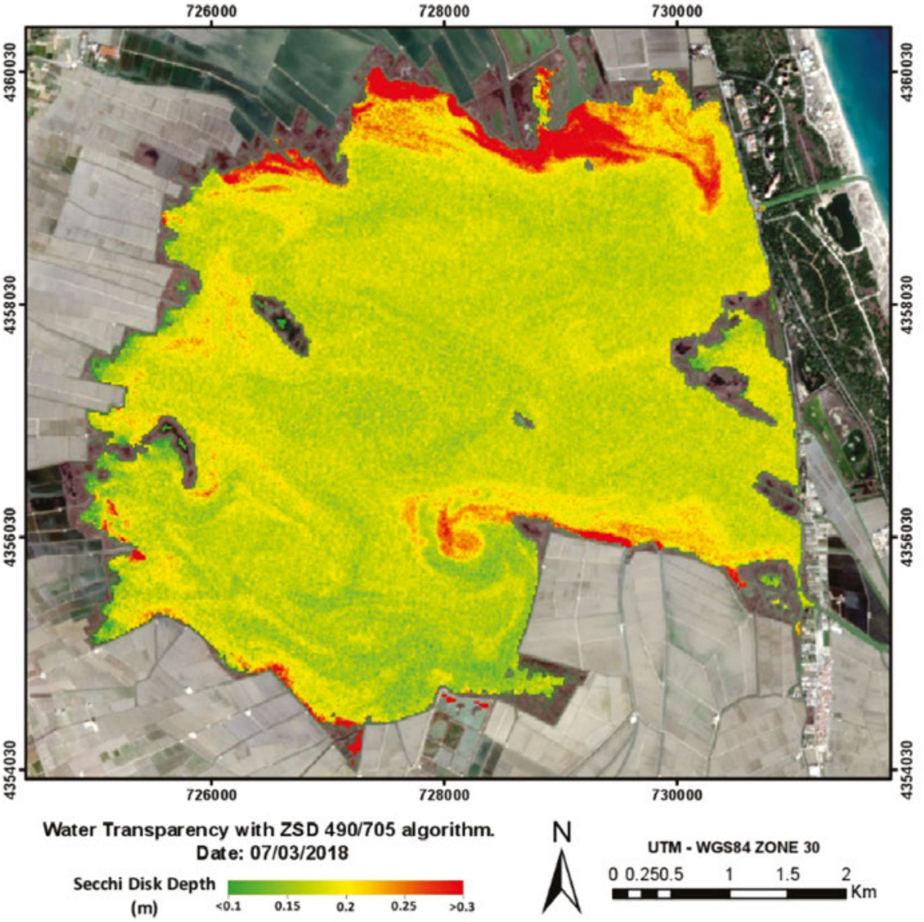

Figure 8. Z_{SD} map by 490/705 ratio in the Albufera of Valencia. S2-MSI image of 07/03/2018. *Mapa de* Z_{SD} *aplicando el ratio 490/705 en la Albufera de Valencia. Imagen S2-MSI del 07/03/2018.*

values have different [Chl-*a*]; this is produced by the presence of NAP giving turbidity to water but without pigments. In this way, Yang *et al.* (2013) also proposed two quasi-analytical algorithms to retrieve total absorption and backscattering coefficients based on a semi-analytical estimation model for clear and turbid inland waters, and these models could be proved in future works. There are many works were Z_{SD} is not well estimated, as Devi Prasad & Siddaraju (2012) in some Indian hypertrophic lakes; Borkman & Smayda (1998) in coastal areas of Rhode Island and Lahtrop (1992) were algorithms derived from samples in Lake Michigan cannot be extrapolated to Yellowstone and Jackson Lakes.

CONCLUSIONS

The accuracy of the algorithms estimated in the reservoirs studied so far is sufficient for most of the applications related to the ecological status assessment. The S2-MSI images showed that they are optimum for performing ecological monitoring on inland waters from space.

It is important to emphasize the relevance of the atmospheric correction method used: Sen2-Cor, which in our case was accurate enough, allows the algorithms to be applied to actual S2-MSI data for routine monitoring purposes, which is the ultimate goal of the ESAQS project. In this context, the next step of the research is to evaluate and validate several atmospheric correction methods for different types of inland water in order to determine the one best suited for achieving the ESAQS objectives.

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Decision support framework to identify lakes that are likely to meet water quality targets if external inputs of phosphorus from agriculture are reduced

Linda May[1,*], Philip Taylor[1], Bryan Spears[1], Jo-Anne Pitt[2], Adrian L. Collins[3], Isabel Corkley[4], Steven Anthony[4], David Skirvin[4], David Lee[4,5] and Pamela Naden[6]

[1] Centre for Ecology & Hydrology, Bush Estate, Penicuik, Midlothian EH26 0QB, UK.
[2] Environment Agency, Station Road, Haddiscoe, Great Yarmouth NR31 9JA, UK.
[3] Sustainable Agriculture Sciences Department, Rothamsted Research, North Wyke, Okehampton, Devon EX20 2SB, UK.
[4] RSK ADAS, Titan 1 Offices, Coxwell Avenue, Wolverhampton Science Park, Stafford Road, Wolverhampton WV10 9RT, UK.
[5] Catapult Energy Systems, 7th Floor Canon House, The Priory Queensway, Birmingham B4 6BS, UK.
[6] Centre for Ecology & Hydrology, Maclean Building, Crowmarsh Gifford, Wallingford OX10 8BB, UK.

[*] Corresponding author: lmay@ceh.ac.uk

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ABSTRACT

Decision support framework to identify lakes that are likely to meet water quality targets if external inputs of phosphorus from agriculture are reduced

About 40 % of European lakes are failing water quality targets for chemistry that have been set under the EU Water Framework Directive (WFD). One of the main causes of this problem is excessive inputs of phosphorus (P) to lakes from their catchments, including those from agricultural sources and wastewater treatment works. This study used WFD monitoring data from England and Wales to develop a method of identifying lakes that are failing WFD water quality targets for good status in terms of total phosphorus (TP) concentrations and whose problems could be solved by reducing P inputs from agricultural sources. A decision support framework is presented that (1) identifies lakes that are failing to achieve good status due to high TP inputs from agriculture; (2) predicts how in-lake TP concentrations are likely to respond to reduced inputs from agricultural sources, and (3) identifies sites where recovery is likely to be delayed by factors that induce resistance to TP mitigation measures. Through a worked example, we demonstrate how a decision support framework can be used to screen lake WFD monitoring data to identify where expensive restoration measures are likely to be more cost effective. We demonstrate that, in general, reducing the external TP load from agricultural sources would be effective only if introduced as part of a more comprehensive programme of restoration measures targeting other sources of TP, such as waste water treatment works, and taking into account lake-specific factors that affect lake response. The outputs from this decision support framework are designed to enable water managers to identify sites where recovery may be delayed by lake specific biological, chemical or physical characteristics, allowing them to manage expectations of the recovery process better by providing realistic timescales.

Key words: Eutrophication, restoration, catchment, runoff, nutrients

RESUMEN

Marco de referencia para identificar lagos que puedan cumplir los objetivos de calidad del agua si se reducen los aportes externos de fósforo de origen agrícola

Alrededor del 40 % de los lagos europeos no cumplen con los objetivos de calidad química del agua que se han establecido en la Directiva Marco del Agua de la UE (DMA). Una de las principales causas es la entrada excesiva de fósforo (P) a los lagos *proveniente de sus cuencas, ya sea por fuentes agrícolas o efluentes de plantas de tratamiento de aguas residuales. Este estudio utilizó datos de monitoreo de la DMA de Inglaterra y Gales para desarrollar un método para identificar lagos que no están cumpliendo con los objetivos de calidad del agua de la DMA en términos de concentraciones de fósforo total (TP) y cuyos problemas podrían resolverse reduciendo las entradas de P de fuentes agrícolas. Se presenta un marco de referencia para la toma de decisiones que (1) identifica los lagos que no logran alcanzar un buen estado debido a los altos aportes de TP de la agricultura; (2) predice cómo las concentraciones de TP en el lago parecen responder a aportes reducidos de fuentes agrícolas, y (3) identifica los sitios donde es probable que la recuperación se retrase debido a factores que inducen resistencia a las medidas de mitigación de TP. A través del ejemplo estudiado, demostramos cómo puede ser usado un marco de referencia para la toma de decisiones, que nos ayude a filtrar los datos de monitoreo de lagos de la DMA y poder identificar dónde son más efectivas las costosas medidas de restauración. También demostramos que en general, reducir la carga externa de TP de origen agrícola solo es efectivo si se introduce como parte de un programa de restauración más completo el cual tenga como objetivo identificar otras fuentes de TP, como por ejemplo las estaciones depuradoras, y teniendo en cuenta factores específicos de los lagos que afectan su respuesta. Los resultados también permiten a las personas encargadas de la gestión del agua identificar sitios donde la recuperación puede retrasarse por las características biológicas, químicas o físicas específicas del lago, lo que permite gestionar mejor las expectativas del proceso de recuperación al proporcionar escalas de tiempo realistas.*

Palabras clave: *Eutrofización, restauración, cuenca, escorrentía, nutrientes*

INTRODUCTION

Many lakes across Europe have water quality targets that have been set under the EU Water Framework Directive (WFD) (European Parliament, 2000). In practice, these are mainly lakes with a surface area of more than 0.5 km^2. Of these, about 40 % (by surface area) are currently failing to achieve good status in relation to WFD water quality targets for chemistry (https://www.eea.europa.eu/data-and-maps/figures/percentage-of-rivers-lakes-groundwater).

Although it is unlikely to be the only pressure that is degrading lake water quality, phosphorus (P) is often the main cause of the cyanobacterial blooms that restrict water use leading to economic impacts. For example, Wolf and Klaiber (2017) estimated that the value of properties in the USA situated within 600 m of waterbodies infested with algal blooms will be reduced by about 22 %. The main sources of P within a lake catchment vary from site to site, with some being dominated by agricultural sources and others by sewage related sources (Comber *et al.*, 2018). To implement the cost effective improvement of water quality in lakes that have been adversely affected by excessive inputs of P, it is important to explore the relative importance of different sources of P and the likely benefits of controlling them. In addition, consideration of factors that may potentially confound ecological recovery following catchment P reduction should be assessed, for example, internal loading (Serano *et al.*, 2017. A growing body of literature is available in which the effects of catchment P reduction and confounding factors are quantified, generally, providing scope to construct a decision support framework to accomplish this.

In England and Wales, about 763 waterbodies have been classified as 'lakes' under the WFD, i.e. lakes with a surface area greater than 0.5 km^2. At the time of this study, many of these were being monitored by the Environment Agency for regulatory and reporting purposes. Although some lakes fail to achieve good status for other reasons, most failures to achieve good chemical status are caused by nutrient enrichment (eutrophication), with agricultural runoff and effluent from wastewater treatment works being the main external sources of these nutrients (e.g. Scottish Government, 2015).

The main aim of this study was to develop a method of identifying lakes that were failing WFD water quality targets for good status in terms of total phosphorus (TP) concentrations and whose problems could, potentially, be solved by reducing TP inputs from agricultural sources. A decision support framework has been developed that (1) identifies lakes that are failing to achieve good status due to high TP concentrations; (2) predicts how in-lake TP concentrations are likely to respond to reductions in TP inputs from agricultural sources, and (3) provides information on where lake recovery could be delayed, or even prevented, by factors that induce resistance to mitigation. These include internal recycling of P within the lake, lake depth, fetch, hydraulic loading, the presence of benthivorous fish and the extent of the littoral zone (Janse *et al.*, 2008).

DATA AND METHODS

Lake and catchment specific values of key parameters

Lake and catchment characteristics

Digital outlines of the WFD lakes and their catchments across England and Wales, and summary information on their size and shape, were obtained from the UK Lakes database (https://eip.ceh.ac.uk/apps/lakes/detail.html). These data included a unique identifier (WBID) for each lake and physical characteristics such as mean depth, surface area, volume and catchment area.

Lakes monitoring data and WFD water quality targets for TP concentrations

The Environment Agency provided water quality monitoring data for 437 WFD lakes from across England and Wales. These data comprised in-lake TP concentrations that had been collected between 2008 and 2014. The methodology for collecting samples and analysing them for TP content was consistent across all lakes. Type or site specific TP boundary values for good/moderate status were also supplied.

Hydraulic loads to lakes

Water flow into each lake was estimated from Standard-period Average Annual Rainfall (SAAR) data at 1 km resolution and actual evaporation data from the Meteorological Office Rainfall and Evaporation Calculation System (MORECS; Hough & Jones, 1997) at 40 km resolution. Mean values for each lake catchment were used to estimate hydrologically effective rainfall (HER). It was assumed that the land type coefficient of 0.7 (i.e. grassy surface) used to calculate MORECS evaporation data was representative of the whole catchment. The calculated value for HER (m) was combined with the catchment area (m^2) to give mean annual runoff into the lake from its catchment. HER falling directly onto the lake surface was calculated using the same data and a similar process, but the land type coefficient was increased to 1 to reflect the higher evaporation levels that would be likely to occur across the lake surface.

Total phosphorus inputs to lakes

Total phosphorus inputs to each lake were estimated using outputs from the SEPARATE (SEctor Pollutant AppoRtionment for the AquaTic Environment; version 2.0) cross sector screening tool (Zhang *et al.*, 2014). SEPARATE integrates information on TP emissions from multiple sources to provide pollutant apportionment information and summarises these estimates for WFD inland water bodies (rivers) across England and Wales. The following sources are included: agriculture, wastewater treatment works, urban diffuse sources, storm tank overflows, septic tanks, combined storm overflows, river bank erosion and direct atmospheric deposition to water.

To estimate TP delivery to lakes from the WFD river catchment data provided by SEPARATE, total catchment TP loads were converted to specific TP loads (i.e. values per unit area of catchment) and then overlain onto the relevant lake catchments. Where the lake catchment polygons and the SEPARATE data intersected, new polygons were created with a unique identifier for each lake (WBID) and the corresponding TP specific load. For each lake catchment, these new polygons and TP loads were aggregated to provide whole-catchment loads. Lake catchments were excluded from the analysis if less than half of the catchment had data coverage or if the total area of the lake catchment was less than 25 km^2, which is the limit of resolution of the agricultural source data currently included in SEPARATE.

Average annual and maximum summer/autumn TP concentrations

The WFD monitoring data were used to calculate average annual and maximum summer/autumn TP concentrations by calculating monthly means, seasonal means, and then annual means. For quality assurance purposes, the monitoring data for each lake were considered sufficient to perform these calculations only if they were available from at least two different months in each season, and at least three seasons within each year. Seasons were defined as spanning three calendar months, with winter, spring, summer and autumn starting in December, March, June and September, respectively; data for December were included in the winter season of the following year. For the purposes of this study, a lake was deemed to have failed WFD water quality targets for TP if the annual mean TP concentration was above the type or site specific good/moderate boundary value for TP provided by the Environment Agency.

Lake pressures and responses

Eutrophication pressures and lake responses, especially in relation to P enrichment, are driven, mainly, by water and nutrient supply, and moderated by lake sensitivity factors such as size, shape and water retention time. Lake responses to changes in TP inputs, hydrology and in-lake TP concentrations, were calculated as detailed below.

Hydrology

The annual input of water to the lake (W_{in} m^3/y) was calculated by summing the HER (runoff) from the catchment (W_{HER} m^3/y) and the annual input of rain falling directly onto the surface of the lake (W_{rain} m^3/y):

$$W_{in} = W_{HER} + W_{rain}$$

The annual outflow from each lake (W_{out} m^3/y) was calculated as the annual input of water to the lake (W_{in}) minus the amount of water lost in evaporation over the surface of the lake (W_{evap} m^3/y):

$$W_{out} = W_{in} - W_{evap}$$

The water retention time of each lake (T_w lake volumes/y) was calculated as the volume of the lake (V_L m^3) divided by the amount of water leaving the lake via its outflow (W_{out} m^3/y):

$$T_w = \frac{V_L}{W_{out}}$$

Total phosphorus input to the lake

The TP input (external loading) to each lake (TP_{in} t/y) was estimated from lake specific TP delivery values derived from SEPARATE, as described above, plus the input of TP from rain falling directly onto the lake surface (TP_{rain}). TP_{rain} (t/y) was calculated as follows:

$$TP_{rain} = \frac{(W_{rain} \times 0.45)}{10^9}$$

with 0.45 mg/m^3 being the average concentration of TP in rainfall across the UK (Neal *et al.*, 2004; Duethmann *et al.*, 2009; Zhang *et al.*, 2014). In addition, the percentage contribution of TP from agricultural sources ($\%\ TP_{ag}$ t/y) was calculated by dividing the TP input from this source (TP_{ag} t/y) by the total TP input to the lake (TP_{in}):

$$\%\ TP_{ag} = \frac{TP_{ag}}{TP_{in}} \times 100$$

Lake response

The response of each lake to external TP loading, in terms of its in-lake TP concentration (mg/m^3), was estimated using a modelling approach similar to that used by OECD (1982). Only 85 lakes had sufficient TP monitoring and loading data to be included in these analyses. Initial results showed that none of the regional models published by OECD (1982) predicted the measured annual mean in-lake TP concentrations (TP_L) of the lakes in England and Wales well. So, the 'combined' model was re-calibrated to improve the goodness-of-fit by adjusting the multiplication and power factors in the model to maximise the r^2 value of a linear regression of the modelled and measured in-lake TP data. This produced the following predictive equation:

$$TP_L = 0.13 \times X^{0.9}$$

where $X = \dfrac{TP_{in} \times 10^9}{W_{in}} / (1 + \sqrt{T_w})$

The relationship between measured and modelled in-lake TP concentrations is shown in figure 1; the r^2 of 0.72 indicates that the model accounts for about 72 % of the variation between the modelled and measured data. Also, it should be noted that the level of uncertainty in the modelled values increases as the in-lake TP concentrations increase. Higher in-lake TP levels tend to correspond to shallow lakes (< 4m depth) and the modelled values are more likely to underestimate the measured values in these systems. It is likely that this reflects the internal loading of P that is common in shallower lakes and is not taken into account in the TP loading data from SEPARATE.

The equation derived from these data was used to predict $_{in}$-lake TP concentrations for all lakes where TPin could be estimated reliably from the SEPARATE data, and where values for W_{in} and T_w were also available (n = 280).

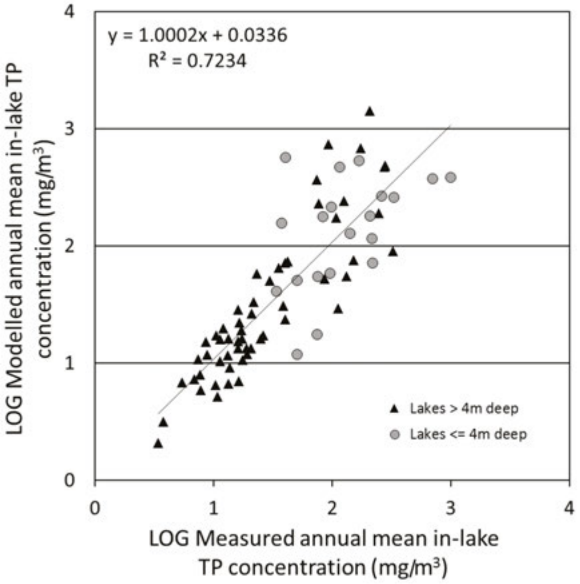

Figure 1. Relationship between measured and modelled in-lake total phosphorus (TP) concentrations for shallow (<= 4m) and deep (> 4m) lakes. *Relación entre las concentraciones del fósforo total (TP), medidas y modeladas, para lagos poco profundos (<= 4m) y lagos profundos (> 4m).*

Critical total phosphorus load

The WFD TP good/moderate boundary value for each lake was used, in combination with the lake response model, to determine the critical TP load below which the modelled in-lake TP concentration would be classified as 'good'. WFD water quality targets for TP concentration were available for only 97 of the 249 lakes that had sufficient data to calculate in-lake TP concentrations.

The estimated critical TP loads for these lakes were compared to the modelled TP loads to estimate the minimum reduction in TP inputs that

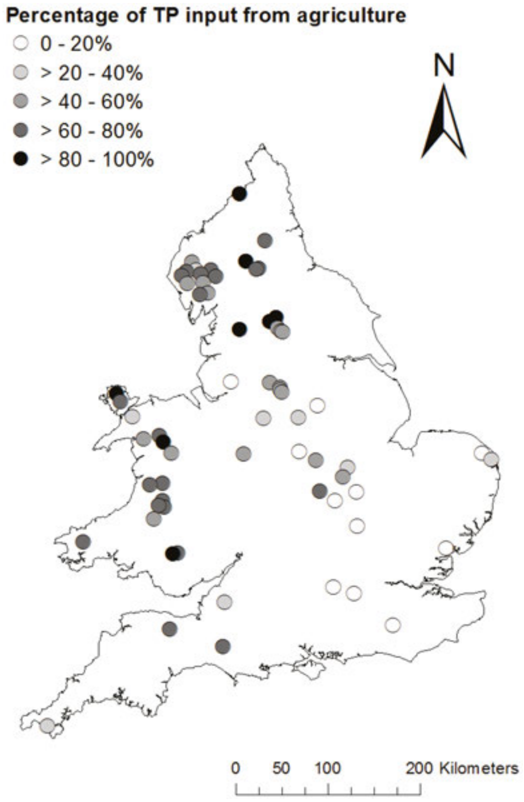

Figure 2. Percentage of the total phosphorus (TP) inputs to lakes in England and Wales that are attributable to agricultural sources, according to SEPARATE source apportionment data; lakes with catchments of < 25 km^2 are excluded (see text for details). *Porcentaje de las aportaciones del fósforo total (TP) a lagos en Inglaterra y Gales que se atribuyen a fuentes agrícolas, de acuerdo con los datos proporcionados por SEPARATE; se han excluido los lagos cuya cuenca es menor de 25 km^2 (detalles en el texto).*

would be required to achieve good water quality. The technically feasible impacts of 0 %, 5 %, 10 %, 15 %, 20 %, 25 %, 50 % and 75 % reductions in TP inputs from agricultural sources on lake water quality were determined for each lake.

RESULTS

The percentage of the TP load to each lake that was originating from agricultural sources was mapped for all lakes in England and Wales where sufficient data were available (Fig. 2). The results showed that agriculture was not the main source of TP inputs to lakes in many areas. In these areas, other sources such as effluent from wastewater treatment works dominated these inputs. Lakes that were found to be failing WFD TP targets for at least good status were compared to those that were predicted to fail under current TP loading conditions using the modelling approach described above. Of the 85 lakes for which comparable data were available, failure to meet TP water quality targets for good status was correctly predicted in 89 % of cases.

The water quality of the 99 lakes for which modelled data were available were classified as 'pass' or 'fail' in terms of meeting good status under different agricultural TP load reduction scenarios. The lake model predicted that, if the TP loads from agricultural sources were reduced, more lakes would pass WFD TP targets (Fig. 3). However, it also predicted that, even with a 100 % reduction in TP load from agricultural sources, 27 % of failing lakes in England and Wales would still not meet the criteria for good status. This is due to other (non-agricultural) sources of TP within their catchments (Zhang *et al.*, 2014).

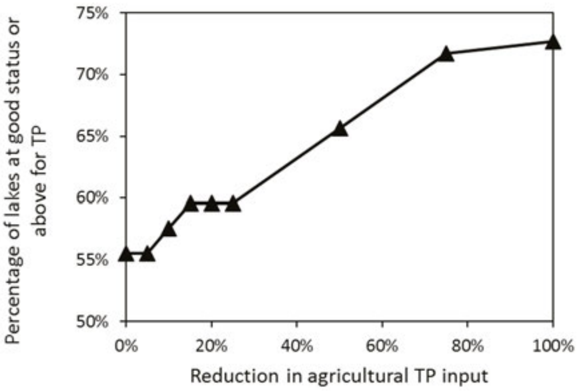

Figure 3. Number of lakes in England and Wales that would achieve WFD good status for total phosphorus (TP) concentrations under different levels of reduction in TP inputs from agricultural sources. *Número de lagos de Inglaterra y Gales que podrían alcanzar el buen estado para la concentración de fósforo total (TP) según la DMA bajo diferentes niveles de reducción en los aportes de TP de origen agrícola.*

To provide spatial detail on failing lakes across England and Wales, these data were mapped. The progressive improvement in lake water quality corresponding to 0 %, 25 %, 50 % and 75 % reductions in TP from agricultural sources is shown in figure 4.

Critical TP loads

Critical TP loads were calculated for the good/moderate WFD boundaries of the 99 lakes for which sufficient data were available. Sixty-four lakes were found to have TP inputs that were above the critical TP load (with 63 being more than 10 % above) and 35 had TP inputs below the critical TP load (with 33 being more than 10 % below).

Using the results to create a decision tree for lake management and restoration

The results summarised above were used to develop the first phase (Phase 1) of a decision tree to help lake managers and regulatory authorities determine which of the lakes that are failing WFD water quality targets for TP would be likely to recover sufficiently to meet those targets if inputs from agricultural sources were reduced. The second phase of this development (Phase 2), aimed to identify lakes where recovery is likely to be delayed, or even prevented, by site specific factors that cause resistance to recovery. These include internal recycling of P within the lake, lake depth, fetch, hydraulic loading, the presence of benthivorous fish and the extent of the littoral zone (Janse *et al.*, 2008).

Phase 1: Identifying lakes that are likely to achieve good status for total phosphorus if inputs from agricultural sources are reduced

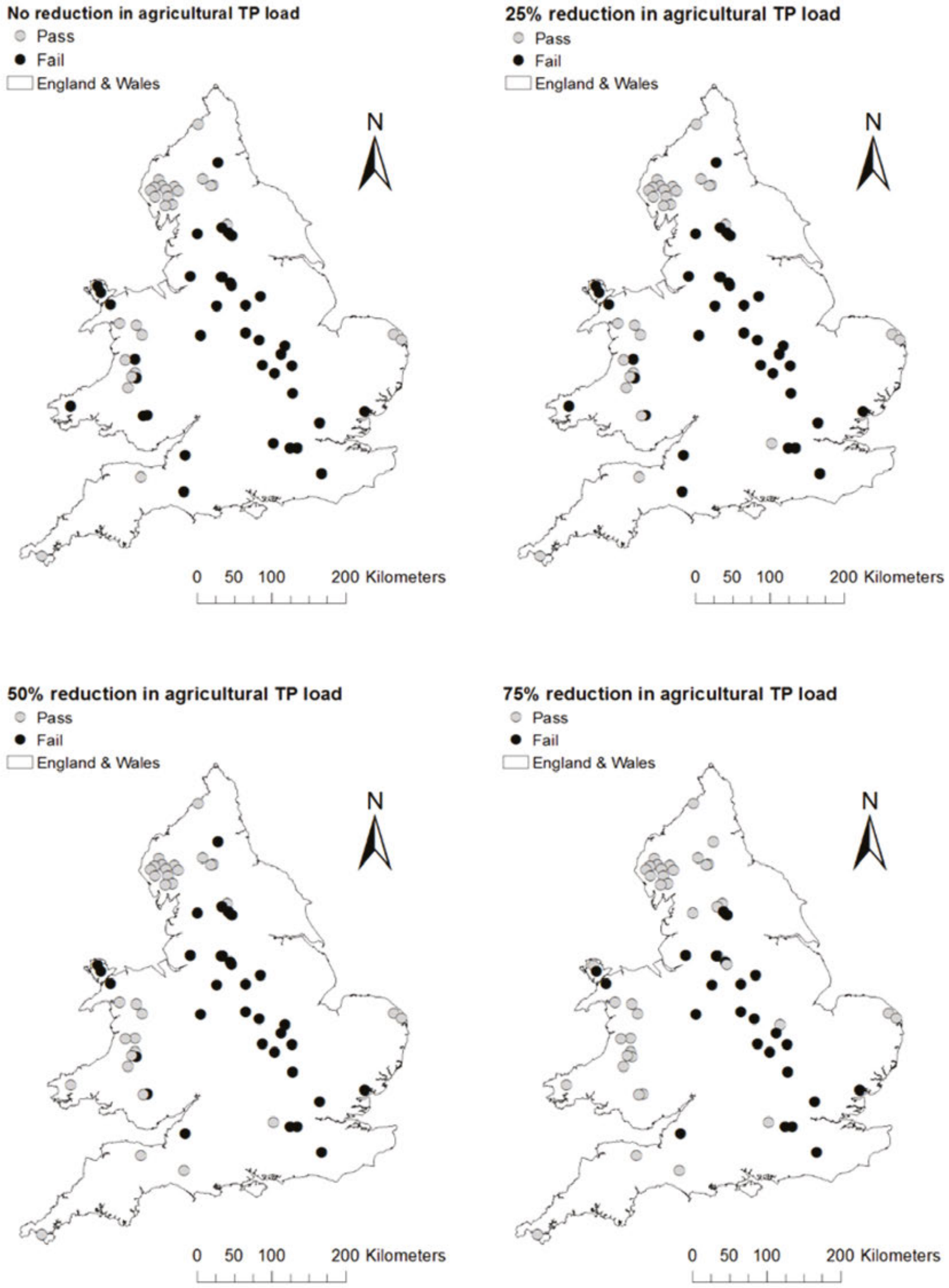

Figure 4. Lakes in England and Wales that are predicted to pass or fail WFD water quality targets based on the good/moderate boundary for total phosphorus (TP) under 0 %, 25 %, 50 % and 75 % reductions in agricultural loads. *Lagos de Inglaterra y Gales que se predice que alcanzarán o no los objetivos de calidad de la DMA, según el límite bueno/moderado para fósforo total (TP) bajo reducciones en 0 %, 25 %, 50 % y 75 % de las cargas agrícolas.*

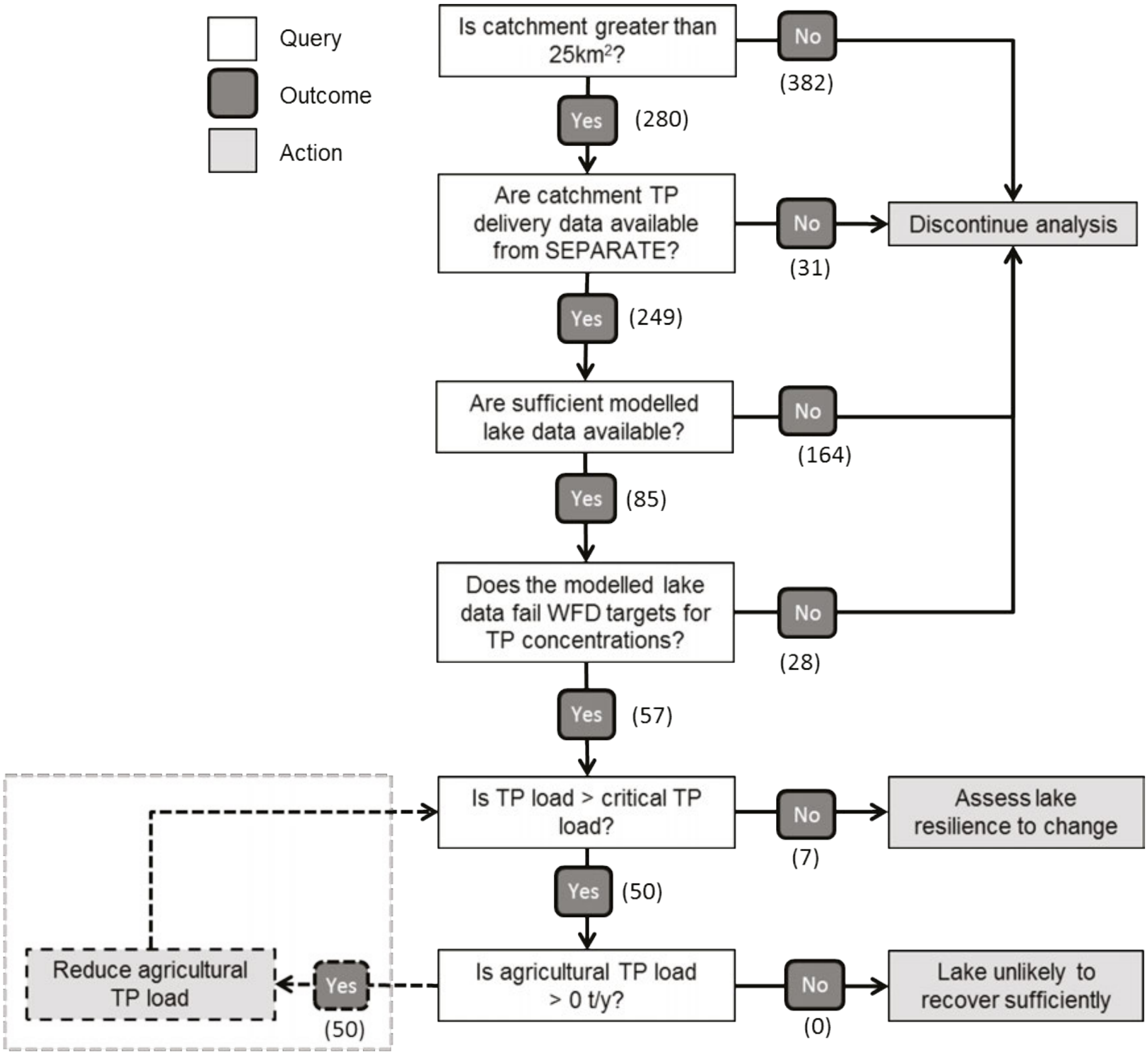

Figure 5. Phase 1 of the decision making process; identifying lakes that are likely to achieve WFD good status for TP concentrations if inputs from agricultural sources are reduced. The number of lakes being passed from step to step is shown in brackets. *Fase 1 del proceso de toma de decisiones; identificación de los lagos que podrían alcanzar el buen estado según la DMA para las concentraciones de TP si se reducen las cargas de origen agrícola. El número de lagos que pasan al siguiente nivel están entre paréntesis.*

A decision tree was constructed to help identify lakes that are likely to achieve good status in relation to TP concentrations following a reduction in TP inputs from agricultural sources (Fig. 5). The first step in this process excludes all lakes that have catchments smaller than 25 km^2 in area because the SEPARATE nutrient load data are unreliable at this scale, especially for agricultural sources. This problem is caused by the underpinning process-based model for the agricultural sector, which uses aggregated statistical input data (Zhang *et al.*, 2014). The second step examines whether TP loading information are available for each lake, with analysis being discontinued for lakes that do not have these data. The third step excludes all lakes for which there are insufficient monitoring data to provide robust information on annual average and summer/autumn maximum in-lake TP concentrations (see Methods section for details). In Step 4, the modelled lake data are compared to the WFD boundary values provided by the Environment Agency to determine whether or not they are failing WFD water quality targets; only those that are failing are passed through to Step 5. Step 5 assesses the likelihood of lakes recovering to at least good status if TP loads from agricultural sources are reduced. This step makes the assumption that any lake where the TP load is at, or below, the critical TP load is likely to recover, eventually. At this stage, any remaining lakes are passed through to Phase 2 of the decision tree (Fig. 6), where the inherent resistance of the lake to restoration efforts is assessed because this is likely to slow the recovery process (see below).

The increasing number of lakes that fall into the 'likely to recover' category as TP inputs from agricultural sources are progressively reduced are shown in table 1. However, it should be noted that, in practice, even a 25 % reduction in TP losses from agriculture would be difficult to achieve, unless uptake of on-farm mitigation measure is high (Collins *et al.*, 2016; Zhang *et al.*, 2017).

Phase 2: Identifying lakes where resilience will delay recovery

The 23 lakes that were assessed as being likely to recover if agricultural inputs are reduced (Table 1) were then examined for their level of resistance to restoration activities. Resistance, which can be caused by the interplay of a range of factors, reduces the rate of recovery even when external inputs of TP have been reduced. Some of these factors (e.g. internal release of P from the sediments, lake depth < 4 m, fetch < 3 km and

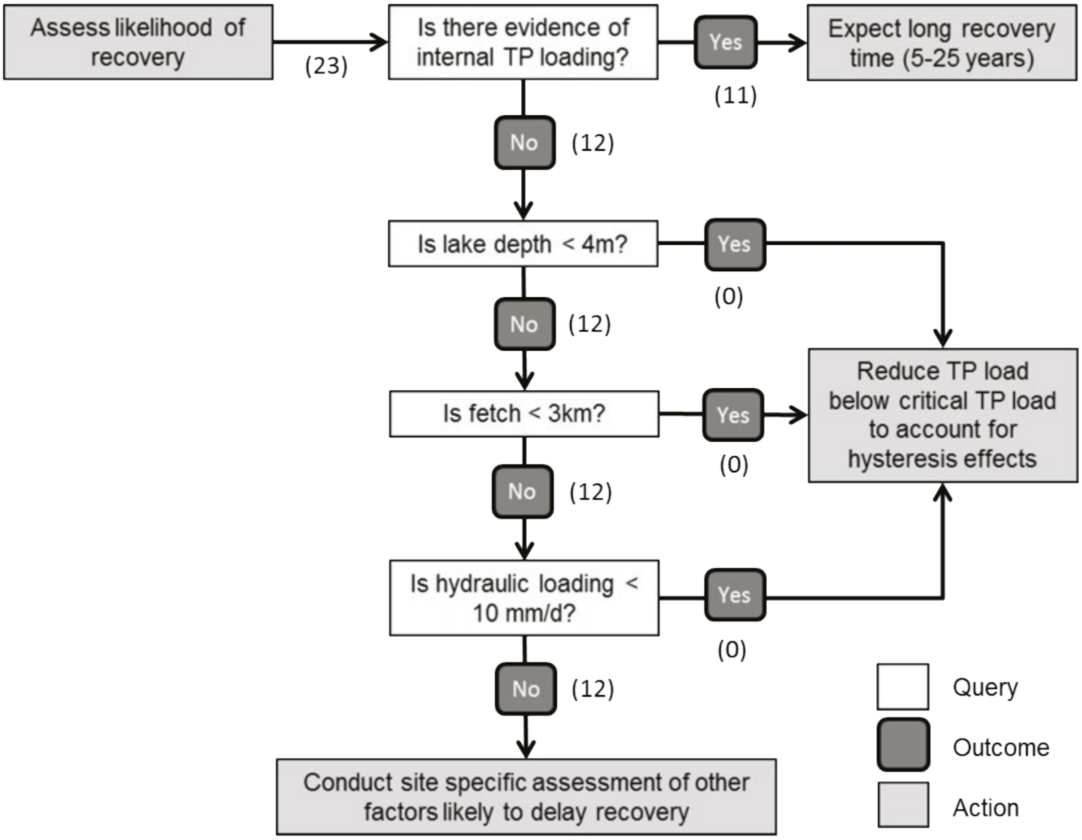

Figure 6. Phase 2 of the decision making process; identifying lakes where resilience to change is likely to delay recovery. The number of lakes being passed from step to step is shown in brackets. *Fase 2 del proceso de toma de decisiones; identificación de los lagos donde la resiliencia al cambio es probable que retrase la recuperación. El número de lagos que pasan al siguiente nivel están entre paréntesis.*

hydraulic loading < 10 mm/d - Janse *et al.*, 2008) were attributed with numerical, or surrogate, values that can be used during the decision making process to screen out lakes that are likely to have a high resistance to restoration efforts (Fig. 6), and therefore long recovery times.

In Phase 2, Step 1 separates out the lakes where recovery is likely to be delayed by internal release of TP from the sediments. This TP is likely to have accumulated within the lake as a result of legacy pollution issues, such as effluent from wastewater discharges or agricultural runoff. The decision making process assumes that high maximum summer/autumn TP concentrations indicate high internal sources, because this is the time of year when large releases of P from lake sediments are most common. The criterion for this is set at summer/autumn in-lake maximum TP concentrations of more than 0.05 mg/l.

Lakes that are deemed not to show evidence of internal P loading are subsequently assessed in relation to other factors that may delay recovery (Steps 2-4). These factors are lake depth, fetch and hydraulic loading. Lakes with a depth of less that 4m tend to have more extensive macrophyte coverage than deeper lakes, which tends to delay recovery. Similarly, those with a maximum fetch of less than 3 km (Step 3), or a with very low flushing rate (Step 4), also tend to recover slowly. Very low flushing rate was approximated to a hydraulic loading of < 10 mm/d. In all of these cases, hysteresis effects are likely to occur when TP inputs are reduced. So, external TP loads may need to be reduced below the modelled critical TP load to compensate for this process during lake restoration.

Lakes that pass through Steps 1-4 then pass into the 'Conduct site specific assessments of other factors likely to delay recovery' action box. At this point, lakes are screened for other potentially confounding factors, such as the density of benthivorous fish or the extent of the littoral zone, which also affect the likelihood and speed of recovery. As these cannot be quantified at national scale, site specific assessments would be required to estimate the likely impact of these on lake recovery processes.

The decision process outlined above identified only 23 lakes as having potential to recover if external TP loads from agricultural sources were reduced (Table 1). Following assessment of the confounding factors shown in figure 6, it was found that only 12 of these lakes were likely to recover quickly (i.e. in less than 5 years). Even then, this level of recovery could be achieved only if TP inputs from agricultural sources were reduced by an unrealistic 100 %.

The use of the proposed decision tree is illustrated using readily available lakes monitoring data from England and Wales, lake characteristics taken from the UK Lakes database (Hughes *et al.*, 2004), and external TP loadings derived from

Table 1. Change in the number of lakes where total phosphorus (TP) load > critical TP load under different levels of reduction in the TP load from agricultural sources. *Cambio en el número de lagos donde la carga de fósforo total (TP) es mayor que el valor crítico de carga de TP, bajo diferentes niveles de reducción de la carga de TP de origen agrícola.*

Reduction in TP input from agriculture	Number of lakes where TP load > critical TP load	Number of lakes where TP load ≤ critical TP load
0%	50	7
25%	50	7
50%	44	13
75%	38	19
100%	34	23

the SEPARATE (version 2.0) load apportionment model (Zhang *et al.*, 2014). The results suggest that, of the 280 WFD lakes in England and Wales with catchments greater than 25 km^2 in area, most had corresponding TP loading data available but only 85 had sufficient modelled data to complete the assessment process. Of these, 57 lakes were found to fail WFD water quality targets for TP concentrations at the good/moderate boundary. In almost all of these cases, the TP load to the lake was predicted to exceed the critical load even if agricultural losses were to be significantly, and unrealistically, reduced. These results suggest that reducing agricultural sources of TP within lake catchments would not be able to improve lake water quality sufficiently to meet WFD water quality targets unless combined with other nutrient reduction strategies. Of the 23 lakes that were identified as having the potential to recover to good water quality if agricultural TP inputs were reduced, 11 showed evidence of internal release of P from the sediments, which could delay recovery for many years.

DISCUSSION

At present, the selection of an appropriate restoration programme for any particular lake, or set of lakes, requires water quality managers to have a detailed understanding of external and internal sources of P to their lake and of the likely ecological responses to those loads (Moss *et al.*, 1996). So, designing and implementing a lake restoration plan can be time consuming and expensive, with positive results only being achieved after long periods of recovery (Carvalho & Kirika, 2003, Jeppesen *et al.*, 2005, Moss *et al.*, 2005, Phillips *et al.*, 2005, Spears *et al.*, 2007). Although difficult at the site specific scale, this situation is even more challenging where there is a need to assess lake restoration requirements and likely outcomes at a regional or national scale.

In this study, we developed a process to help water managers assess the extent to which failing lakes across a large area would be likely to meet WFD water quality targets for TP if inputs from agricultural sources, alone, were reduced. Our approach builds on a decision making process that was originally developed to identify failing lakes that would be suitable for another management approach, namely the control of internal P recycling using a geo-engineering approach (Spears *et al.*, 2011). Our new approach is illustrated using WFD monitoring data from England and Wales.

The decision making process that we have proposed allows operational monitoring data from a large number of lakes to be summarised, screened, and categorised into suitable management categories on a national scale. The process uses decision criteria that are based on scientific evidence, thus making research results available to lake managers and regulatory authorities in a readily usable form and, thereby, reducing the level of uncertainty in the outcome. In particular, a Phase 2 decision tree has been added to that proposed by Spears *et al.* (2011). This enables candidate lakes to be assessed for their resistance to restoration, as this may affect the timing and cost-effectiveness of any management activity that is undertaken.

One of the limitations of earlier versions of the decision tree was its complete reliance upon sufficient lake monitoring data being available for all of the lakes being screened. In contrast, this new version takes a more modelling based approach. This allows a patchwork of monitoring data to be used to calibrate models that can be used to address gaps in data at national scale.

Although this study presents a large step forward in the development of a decision support framework for data managers, it also demonstrates that the data collected by routine monitoring surveys are often unsuitable for this purpose. While they show which lakes are degraded/degrading, they are of limited use in separating catchment inputs of P from those resulting from internal release from lake sediments. Further research is needed to address this issue and enable restoration measures to be applied cost effectively.

CONCLUSIONS

The proposed lake screening tool, which is based on lake characteristics and monitoring data that are routinely collected for regulatory and reporting purposes, can be applied at national scale to assess the suitability of lakes for a wide range of management techniques. It also provides a science based assessment for use by non-specialists, although it should be noted that this tool is not a substitute for the detailed understanding of individual lakes and lake processes that are required before embarking on costly restoration measures at any particular site. No national scale datasets include the site specific detail that may be critical to the success of restoration activities.

The small number of lakes passing through the screening process illustrates how confounding factors are often present when trying to assess why a lake has failed to meet water quality targets. Although data availability had the greatest impact on the effective use of earlier versions of this lake screening tool, this problem has been addressed, to some extent, in this version by incorporating a simple lake model into the decision making process.

The dataset used in this study demonstrates that there a very few lakes across England and Wales that could be restored to good water quality by reducing external inputs from agricultural sources alone. More generally, reducing the external TP load from agricultural sources would be effective only if introduced as part of a more comprehensive programme of restoration measures targeting other sources of TP, such as waste water treatment works, and taking into account site-specific factors that affect lake response. These include the extent to which historical nutrient inputs that have accumulated in lake sediments will be released into the water column during the recovery process.

ACKNOWLEDGEMENTS

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The opaque lagoon. Water management and governance in l'Albufera de València wetland (Spain)

Anne Jégou[1,*] and Carles Sanchis-Ibor[2]

[1] Laboratoire ThéMA, Département de Géographie, Université de Bourgogne, 2 boulevard Gabriel, 21 000 Dijon, France.
[2] Centro Valenciano de Estudios del Riego, Universitat Politècnica de València, Camí de Vera s/n, 46022 València, Spain.

[*] Corresponding author: anne.jegou@u-bourgogne.fr

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ABSTRACT

The opaque lagoon. Water management and governance in l'Albufera de València wetland (Spain)

The Albufera de Valencia is a complex anthropogenic waterscape, constructed by different social groups over centuries. Today water management in the protected wetland remains opaque, much like the water in the eutrophic lagoon. Four major organizations manage this natural heritage, which remains torn between nature conservation and rice growing, in a jigsaw of overlapping jurisdictions and crisscrossed visions. Water management in this socioecological-system is complex and contested, and takes place within a changing scenario due to recent variations in the water quality and quantity budgets. This paper analyses this changing socio-ecological system based on interviews with stakeholders, and advocates for a new model of management based on shared governance, mediation and transparent data.

Key words: water management, governance, protected area, sustainability, Albufera de València, ecological flows

RESUMEN

La laguna opaca. Gestión y gobernanza del agua en l'Albufera de València (España)

La Albufera de València es un humedal sometido, desde hace varios siglos, a una acusada influencia antrópica. Hoy día se halla en un estadio eutrófico, y la falta de transparencia de sus aguas es comparable a la opacidad en determinados ámbitos de su gestión, en la que chocan los intereses contrapuestos del conservacionismo y el productivismo, en un entramado de jurisdicciones solapadas y visiones contrastadas. La gestión del agua en este sistema socio-ecológico es compleja y conflictiva, y tiene lugar en un escenario cambiante debido a las variaciones en el balance de recursos hídricos y la calidad de las aguas. Este artículo analiza este sistema socio-ecológico a partir de entrevistas con diversos usuarios y agentes, y propone un nuevo modelo de gestión basado en una gobernanza compartida, la mediación y la transparencia informativa.

Palabras clave: *gestión del agua, gobernanza, espacios protegidos, sostenibilidad, Albufera de València, caudal ecológico*

INTRODUCTION

The Albufera is a coastal freshwater hypereutrophic lagoon 10 km south of Valencia (Spain). The lagoon (2320 ha) is surrounded by an arch of marshlands where rice is grown (16 000 ha), and separated from the Mediterranean Sea by a forested sand barrier. The case of the Albufera illustrates the complexity of water management in some Mediterranean wetlands where the connection between irrigation systems and palustrine ecosystems creates an amalgam of contrasted visions, goals, functions and values, generating enormous difficulties to define shared strategies for sustainability.

This work pays tribute to Professor Maria Rosa Miracle. She made the Albufera a preeminent subject of research, developing seminal works on its hydrobiology and guiding the action of numerous younger researchers (Serra *et al.*, 1984; Garcia *et al.*, 1984; Soria *et al.*, 1987; Miracle *et al.*, 1987; Vicente & Miracle, 1988; Alfonso & Miracle, 1990; Vicente & Miracle, 1992; Miracle & Vicente, 1993; Romo & Miracle, 1993, 1994, 1994a; Soria *et al.*, 2001, 2002, 2005; Oltra *et al.*, 2001; Miracle & Sahuquillo, 2002; Romo *et al.*, 2008; Miracle, 2012; Marco-Barba *et al.*, 2013; Onandia *et al.*, 2014, 2015). Beyond this outstanding scientific contribution, she was also a passionate and vocal advocate for the protection and recovery of the Albufera.

Throughout the last two decades, on several occasions, Professor Miracle told the authors that the human-induced hypereutrophic stage of the Albufera could be reversed in the space of a few years. It "just" depended on how water management was handled: "*It's only a problem of water flow regulation, with clean water. I think we have technology also to clean water. Aquatic ecosystems are very grateful: it just took one day or two for reproduction of seaweed. If we give clean water, good seaweeds, then macrophytes come out*" (Interview 2014-8 see supplementary information at http://www.limnetica.net/en/limnetica)1. The Albufera waters, unfortunately, are still not transparent. The lagoon got dark during the 1970s, as a result of the uncontrolled industrialization and urbanization of the surrounding areas, and from then on, only sporadic short winter clear phases have taken place. Water management is also opaque. Information loopholes, overlapped institutions and contested visions are, among others, responsible for this murkiness, which dramatically hinders ecosystem recovery.

This opacity is investigated in this paper. How is water managed in the Albufera Natural Park? How can the management be made transparent and consistent? This paper addresses environmental geography through an empirical approach based on numerous interviews and on the review of local documentation and events. It begins with the historical construction of a complex water system and the way it operates today. The purpose is to understand a territory that is "on hold", where water management is a jigsaw made up of multiple stakeholders. The final part of the paper suggests some changes for the future, in order to work towards the good management that professor Miracle claimed for.

METHODOLOGICAL AND CONCEPTUAL FRAMEWORK

This paper deals with environmental geography through a territorial approach linking stakeholders and ecosystems. Environmental geography aims at all-round thinking involving both political and natural dynamics. This approach is based on the ethical values of ecological centrism: humans surrounded by their biosphere and belonging to it (Larrère, 2010), and managing ecosystems holistically. Two concepts are related to this integrated vision. First, the notion of "médiance", a French neologism created by Augustin Berque in 1990, an orientalist geographer and philosopher, to understand the intricate and inseparable relation between a society and its "milieu", its natural environment (Berque, 1990). In continuation of this concept appeared the "mésologie" as a science for the analysis of the humans' "milieux" (Berque, 1996). This link between social and ecological environments has also been explored by Berkes and Folke (1998, 2006), who coined the concept socio-ecological system (SES) to provide a holistic framework to understand and strengthen ecosystems management. This has opened a field of research for the analysis of ecosystems resilience and robustness (Folke, 2006; Anderies *et al.*, 2004;

2013; Janssen *et al.*, 2007). Both eco-social perspectives provide the appropriate conceptual framework to analyse complex interactions in ecosystems such as the Albufera wetland.

The main source of information of this research consisted of semi-structured interviews conducted as part of a comprehensive approach (Kaufmann, 1996), during two periods of fieldwork in 2004 (10 weeks and 49 interviews) and 2014 (11 days and 18 interviews), held in Spanish with stakeholders from 25 different organizations. Some of the interviewees were contacted in both periods, providing perspective on how the Albufera had changed over 10 years. For each survey, participants were asked about the current state of the wetland, management practices and expected evolution. For confidentiality reasons, we have not used their name when quoting them but we use their number in classification for the excerpts translated for this paper: interviews 2014-1 to 6 are political institutions, 2014-7 is a local councillor, 2014-8 to 10 are scientists, 2014-11 is a farmer, 2014-12 and 13 are companies, 2014-14 to 17 are NGOs and finally 2014-18 is a media.

We also used information from another 30 interviews with key agents of the recent history of the Albufera that were recently published by the *Assut Foundation* (Llorens & Dies, 2017), and the unpublished documentation and video files of the Symposium "*L'Albufera Ara*" that took place in Valencia in December 2016, featuring experts, managers and researchers working in the Albufera throughout the last decades. Other information sources consulted include legal or educational documents and the daily local press.

STATES AND CHANGES IN THE CONSTRUCTION OF THE ANTHROPOGENIC SYSTEM

The Albufera can be thought of as a complex anthropogenic system (Fig. 1), an artefact of

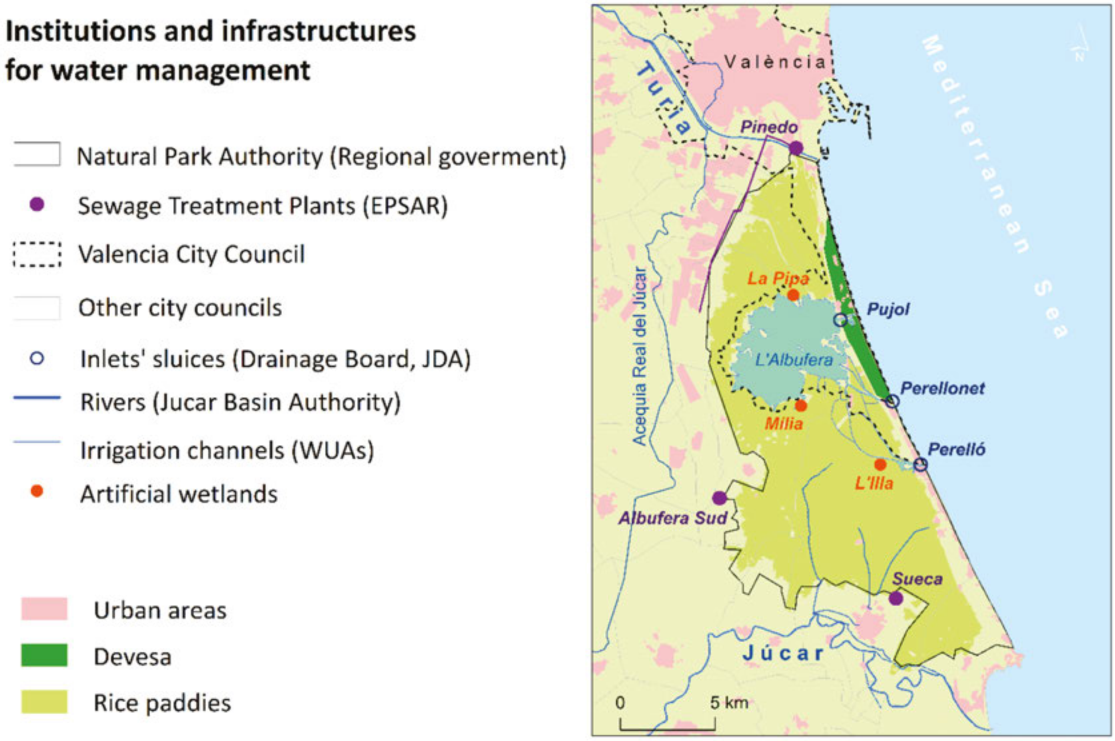

Figure 1. The Albufera complex jigsaw of jurisdictions and infrastructures. *El complejo entramado de jurisdicciones y de infraestructuras en la Albufera.*

nature and culture that has resulted from a long material and immaterial mutual interaction. The different waterscapes over the centuries have reflected the identities, ideas and technologies of the dominant stakeholders, and can be conceptualized as different "médiances", each of them attached to a particular water management system and to different forms of governance (Sanchis-Ibor *et al.*, 2008). From Antiquity until the 17th century, the Albufera was a large brackish lagoon (Rodrigo *et al.*, 2009; Marco-Barba, 2009), connected to the sea through a regulated 200 m wide inlet. The spectrum of fluctuations of the water table was controlled by the local fishing community, closing and opening the inlet and artificially introducing water from the Xúquer and Túria rivers when it was necessary to raise the water level (Sanchis-Ibor, 2001).

In 1760 rice farmers took the control of the inlet. With the support of the Crown, they drained the marshes and irrigated the new lands (García Monerris, 1983). In the following decades, farmers stopped using the natural inlet, which had become disconnected and far from the lagoon due to the reduction in the wetland's size. They opened three new canals to drain the lagoon, installing sluices to close the flow from the sea. The lagoon came to be supplied exclusively with abundant fresh water through irrigation channels from the rivers, primarily from the *Acequia Real del Júcar*. Salinity decreased, and the wetland became an oligotrophic freshwater lagoon (Sanchis-Ibor 2001; Rodrigo *et al.*, 2009; Marco-Barba, 2009). 102 Small polders (the so-called *tancats*) were built to drain the lands located below the lagoon water level (5 200 hectares).

During the second half of the 20th century, the lagoon was transformed into a third waterscape as the area came to be dominated by residential and manufacturing developments. This unplanned urban sprawl spread from València city, mostly affecting the west and northern shores. Abundant wastewater was discharged into the Albufera via the network of irrigation channels, and farmers used increasing amounts of fertilizers and pesticides. This transformation peaked from 1970 to 1980. The Albufera became heavily polluted, receiving enormous quantities of phosphorus and nitrogen (and even heavy metals) and in the end became highly eutrophied, reducing the number and variety of macrophytes and fish species (Carrasco *et al.*, 1972; Blanco, 1984; Blanco *et al.*, 2003; Soria, 1987; Vicente & Miracle, 1992; Miracle & Vicente, 1993). In this transitional period, the coastal sand barrier (the Devesa) was also partially urbanized (1967–1974) with a touristic development project launched during the Francoist dictatorship, that threatened the conservation of the ecosystem.

During the 1970s and 1980s, the degradation of the ecological conditions of the lagoon took place simultaneously to important institutional and social changes. The Albufera (its coastal lagoon, its Devesa and its rice crops) came to be seen as natural heritage. The "*Tot el Saler per al poble*" social movement claimed the public ownership of the Devesa. Both the lagoon and the sand barrier were under the jurisdiction of the Valencia City, so the City Council stopped the development project, started controlling the access to the coastal forest, and in 1980 created a technical service (OTDA) to restore the sand dunes and the pine forest the condition they were in prior to the urbanization project. A few years later, the new Autonomous Community of Valencian Country created the Natural Park of the Albufera of Valencia (1986) to give a legal identity, ensure conservation, and provide autonomous legal standing to the area.

Since the creation of the Natural Park, the management of the area has been clearly tugged in two directions: between agricultural and economic exploitation on one side and ecological conservation on the other, creating two different "frames" according to Mieke Hulshof's structural approach (Hulshof, 2012; Husholf & Vos, 2016). Too frequently, the two directions come into opposition. For example, just after the park was created, farmers' organizations and other businessmen created an association to fight against the legal restrictions imposed by the conservation institution. They took the case to court, and in 1992, the decree creating the Natural Park was annulled (however, it was reapproved in 1993). At other times, the two directions coincide with negotiations or empirical solutions, such as the use of pheromones to fight against plagues.

These two directions correspond not only to two different social groups but, most of all, to different outlooks and relationships with nature. Nature conservation is perceived as a severe restriction by numerous farmers, whereas the ecologist organizations blame some farming practices for some detrimental changes to the wetland ecosystem (shorted flooding periods, low water levels, concrete lining of canals, etc). However, when they are interviewed, both groups share the mutual understanding of a common necessity to protect the wetland. Ecologists defend the critical role of rice paddies in the conservation of wetland fauna, whereas farmers acknowledge the added value that the wetland bestows to their rice production.

The lack or insufficiency of economic compensation for the ecosystem services provided by rice farmers hinders this convergence of interests. The recent reforms to the Common Agricultural Policy (CAP) specifically addressed these agro-environmental systems, have given specific economic support to sustainable farming practices developed within the Nature 2000 network. However, the Albufera farmers have publically threatened the administration with protests against the CAP agro-environmental subsidies if this aid involves forbidding certain practices, such as burning the straw after the rice harvest (Llorens & Dies, 2017). The economic compensation is not enough to abandon unsustainable practices.

INSTITUTIONAL FRAMEWORK WITH CRISS-CROSSED TOOLS AND JURISDICTIONS: WHAT ARE THE GOALS FOR WATER MANAGEMENT?

The Albufera of the 1960s is unanimously considered by all the interviewees as the best state of the waterscape, while that of the 1980s saw the worst years, with dark water and massive fish death episodes in the irrigation channels and lagoon shores. One of the most important points to debate is the benchmark for ecological quality. Most stakeholders refer to the Albufera of 1960s as a model to restore. Hulshof and Vos (2016) called it the “idyllic landscape frame”. This opinion is shared not only by environmentalists but also by farmers and fishermen. The period is associated with bucolic collective memories, such as swimming in the lagoon, eating fresh prawn cones, and the availability of drinkable spring water in the middle of the lagoon.

To return to the pristine conditions of the 1960s is also the management goal settled by the Júcar Basin Authority (*Confederacion Hidrografica del Júcar*), acknowledged after a participatory process that took place in 2004 (CHJ, 2004; 2016), which was also the basis for an ambitious research program (*Albufera Sostenible*) and for the planning of restoration projects in the hydraulic system. The *Albufera Sostenible* program established an exhaustive list of waterworks. This mission included the interception and treatment of the sewage systems surrounding the Albufera (mainly through the new *Colector Oeste* pipe), the construction of several storm tanks to capture polluted rainfall, and the creation of several artificial wetlands to act as green filters to renaturalize the treated wastewater (Martín, 2008; Mondría, 2010).

Unfortunately, the overlapping structure of administrative jurisdictions and responsibilities in the Albufera does not contribute positively to the facilitation of ecosystem management. Four main institutions act as decision makers, and share in the jigsaw of jurisdictions to create a “disorganized” (Interview 2014-4, see supplementary information at http://www.limnetica.net/en/limnetica)[2] system of water management. These are: the Autonomous Community Authority (*Generalitat Valenciana*), the Devesa-Albufera technical department of the Valencia City Council (the *Oficina Técnica Devesa-Albufera*), the Júcar Basin Authority (*Confederacion Hidrográfica del Júcar - CHJ*), and the Drainage Board (*Junta de Desagüe, JDA*).

Two of these bodies are more publicly visible, concerned mainly with ecosystem conservation. Some of their powers overlap, as do the locations that they manage:

- the Autonomous Community Authority (*Generalitat Valenciana*), through two institutions: one for natural areas management (Albufera Natural Park Authority) and one managing water treatment plants (EPSAR).
- Valencia City Council, the owner of the Devesa and the lagoon through its technical department: the *Oficina Técnica Devesa-Albufera*

The other two institutions are less visible but ultimately more powerful, and both are specifically concerned with water management.

• The Júcar Basin Authority (*Confederacion Hidrográfica del Júcar - CHJ*), belonging the Ministry of the Environment, is responsible for water management in all of the Albufera hydrographic basin.

• The "Drainage Board" (*Junta de Desagüe, JDA*) is a government board elected by the all the farmers of the *tancats* area (polders), where all the municipalities sharing the *tancats* are represented, but the presidency is permanently held by the Valencia City Council. It was created at the beginning of the 19th century to control the water level of the lagoon and to facilitate drainage works (Sanchis, 2001). It controls the water level in the lagoon and rice paddies by means of sluice control in the three outlets (*goles*) and a pumping station at the Pujol outlet. The Spanish Water Law does not acknowledge this privative management of a public water body, but the JDA argues that the Albufera is a private water body owned by the Valencia City Council.

The coastal lagoon, within its medieval boundaries corresponding to the rice fields, has been protected since 1986 by the Autonomous Community Authority. It was the subject of two protection plans before the current Pilot Plan for Use and Management was approved in 2008, after eight years of negotiations. But a part of that plan is not yet in action under a legal ruling. This part concerns different areas of protection, as construction threats are pressing on the coast. Moreover, the plan currently in force does not really deal with rules about water management. A new plan was outlined in 2013, including recommendations for water management, but it is also on hold. So, the park still waits for legal documentation to define water uses and protection levels.

Since the beginning of the last financial crisis the Park Authority has encountered great barriers to the mobilization of economic and human resources. In contrast, the technical department of Valencia City has maintained its resources with the support of European funds. During the last decade, this delineation has created a contrast between the areas managed by the Park Authority and the areas managed by the City Council (Jégou, 2015), which partially explains the difference in ecosystem recovery rates between the Devesa and the lagoon and rice paddies, much more favourable to the dune barrier landscape.

As the Spanish central government owns all the water bodies (according to the 1986 Water Law), the CHJ allots water quantities to users, particularly to irrigator's communities. The central government is also in charge of the construction of the main infrastructures of the sewer system.

Although water levels in the lake are controlled exclusively by farmers, other stakeholders have an interest in water management: fishing communities, hunters, touristic residents, ecology associations and foundations. Two associations are particularly active: *Acció Ecologista-Agró*, which has defended an interest in the park for years and is able to bring legal action; and the Community of Fishermen of El Palmar. Both groups are significantly affected by the fact that agriculture is the only criteria to control the water levels and the communication with the sea.

Information has not been publicly available about how water is managed within the lagoon, that is to say, how the water level is adjusted depending on the times of year, except that it is adapted for rice farming. The Drainage Board (JDA) does not reveal its timetable and has acted arbitrarily during the last decades. This has been tolerated by Valencia City Council until recently, and has generated conflicts with the other administrative bodies. In 2016, a professional fisherman sued the JDA for drying the drainage canals. In 2017 the attorney's office ordered that control should lie with the Autonomous Community Authority, but it is not yet clear how this is going to be implemented. Therefore, on paper, it is now possible to ask for information about how the lagoon is regulated from the JDA.

This complicated situation for water management led Mieke Husholf (2012) to conclude that the Albufera is "stuck in fragmentation". Water management in the lagoon is disconnected from protected area management: all the parameters are handled for rice growing, such as the water level in the lagoon and the water allotted to the area. From an institutional perspective, this is the most evident dysfunction of this socio-ecological system. According to common-pool resources theory (Ostrom, 1990) collective management of shared natural resources requires following a reduced number of basic rules that are not respected in the Albufera. These rules include participation of all the users in decision-making processes affecting the collective wealth, which is also intrinsic to democratic societies. The current situation fails to conform to the Spanish legal framework, which considers all the surface water bodies as public wealth. It is also far from the spirit of the European Water Framework Directive, which advocates for the representation of all users in the management of water. In the Albufera, despite the collective societal interest, the water levels are managed as a private water body. This could be considered inertia or the remainder of a former "médiance", in which rice production was the dominant activity that configured and defined the wetland system. This "médiance" does not exist anymore.

MANAGING WATER QUALITY: PURSUING TRANSPARENCY

National and regional administrations made important investments in water treatment in the 1980s and 1990s to improve water quality in the ecosystem. Chlorophyll-*a* levels significantly decreased during the 1990s, and are still slowly decreasing, since 2016 even below the hypereutrophic levels.

However, the water quality has not improved sufficiently to meet sound ecological standards and this progress is not enough for conservationists. "*The improvement is stagnant. […] For a decade, we have been in a period of improvement but we are stagnant […]: the process is slower in an ecosystem so altered*" (Interview 2014-4, see supplementary information at http://www.limnetica.net/en/limnetica)[3]. If wastewater treatment plants and sewage pipelines were built around the park, with some having tertiary processing capable of removing some of the phosphorus and nitrogen, it would be possible to absorb all the urban water of the system.

However, the sewer transportation system remains inadequate: it is incomplete on the west shore of the Albufera and it cannot process stormwater during downpours. The tertiary treatment is not fully efficient, even not in operation or existent in some places, and the treated water contains an excess of phosphorus that feeds chlorophyll production. Moreover, there are still some irrigation channels in the northern area receiving sewage from some urban areas, and directly discharging pollutants into the lagoon.

Green filters have been used in the lagoon since 2008 through three artificial wetlands (*la Pipa*, *Mília* and *l'Illa*) in place of rice field *tancats*. *La Pipa* was designed to restore part of the wetland which was going to be affected by a failed project of ravine channelization. It has produced significant educational and scientific results. *Mília* and *l'Illa* were designed to clean and renaturalize the water after initial processing in a treatment plant, as a part of the *Albufera Sostenible* project. They have never been connected to the sewage treatment plants and only filter water from the drainage channels of the irrigation system.

Clear phases were frequent at the beginning of the 21st century but they have been rare during the last decade. Fortunately, during the last two years a limited but significant bloom of macrophytes (*Potamogeton pectinatus*, *Najas marina*, *Ceratophillum demersum*, *Myriophyllum spicatum*, *Potamogeton crispus*) has taken place in Spring and Autumn, interrupted by massive mortalities in Winter and Summer. These changes evince the lagoon's capacity of self-regeneration, which was postulated on several occasions by professor Miracle. However, the lagoon mat-sediments trap significant levels of toxic heavy metals, and other researchers fear that an improvement of water quality and a decrease in pH could activate all the heavy metals accumulated at the bottom of the lagoon (Martín-Monerris, 1998). This has been defined by some experts as a Damocles' sword over the wetland (Llorens & Dies, 2017).

Flushing operations during clear phases may provide a tool for water renovation. According to some interviewees, this requires a sound and coordinated management, integrating all the system users: "*We don't think it could be resolved with less water, but the management is lacking consultation between authorities: for example,* *there are two crucial periods for the lagoon, which are well known. If the Albufera would have enough good water during those crucial periods, we could achieve recovery of part of the submerged meadows. For that, it's necessary to involve the rice farmers and environmental stakeholders of the area*" (Interview 2014-5, see supplementary information at http://www.limnetica.net/en/limnetica)[4].

The relationship between water quality and quantity is an important issue. Pollution concentrates when water quantities are low. Lagoon water was renovated 21 times per year between 1971 and 1981, but only 10 times per year between 1999 and 2009 (Mondriá, 2010). This change is, together with the temporary suspension of the construction of the sewage transportation networks, the major factor explaining the very limited progress in water quality during the last years. This connection between quantity and quality tends to be regularly mentioned by environmental associations and researchers (Husholf & Vos, 2016).

WATER QUANTITY DILEMA

During the decade prior to the creation of the Natural Park, the lagoon annually received 300 hm^3 from the Júcar River, and 200 hm^3 from the Turia River, wastewater and other sources: a total of 500 hm^3. 20 years later, between 2000 and 2009, the Júcar River only provided more than 100 hm^3 during the wet years and despite the increase in treated wastewater, the total water input has never reached 300 hm^3 (Mondriá, 2010). The wetland has lost the 40 % of its water input in 30 years, and treated wastewater has increased significantly, whereas "natural sources" proportionally decrease. Is this the start of a new médiance?

Water quantity has decreased because the lagoon is the last water body in the Júcar Basin, in geographical order, following the river flow, before the waters reach the sea. The wetland is affected by recurrent drought periods, but beyond this temporary scarcity, the Albufera is threatened by the structural deficit of the Júcar basin, estimated to be 195 hm^3 per year in the current Basin Water Plan (2015-2020). The Júcar River district is, in fact, a well-known case of basin closure (Molle *et al.*, 2010; Avellà *et al.*, 2014) with a substantial imbalance between "paper water" (official allocations) and real water, due to the recent disproportioned expansion of uses and the overestimation of basin resources in the water planning cycle of 1998 (CHJ, 1998). This quantitativist perception of water is not accepted for some stakeholders: "*They consider the river as water. It's the same to say the forest is only timber, but the river is obviously not only water*" (Interview 2014-8, see supplementary information at http://www.limnetica.net/en/limnetica)[5].

According to the recent official water basin plans, the water allocated to the lagoon has increased in the last two decades. The 1998 Basin Plan estimated 100 hm^3 as water necessities for the Albufera. Ten years later, the Basin Plan of 2008 allocated 167 hm^3, the percentile 95 % of the historical series estimated in the study for sustainable development of the Albufera (CHJ-TYPSA, 2004). The current Water Basin Plan (2015-2020) has allocated 210 hm^3, adding some winter flows from Júcar and Turia basin to the historical surface and groundwater inputs, and to the irrigation return flows.

Does this mean that the Albufera has been receiving an increasing volume of water during the last two decades? Unfortunately, not. Or at least this is the perception of most of the agents involved in the management of the Albufera. Most of them express the opposite opinion. "*During one of the last meetings, there was a consensus: the Albufera gets less water and needs quality water from the Júcar. This is established by the law*" "*All is regulated on the paper but is not achieved*" (Interview 2014-5, see supplementary information at http://www.limnetica.net/en/limnetica)[6]. And the gauging stations installed since 2014 by the regional government in the main 15 irrigation channels never have measured more than 100 hm^3 per year.

In any case, there is no way to easily check the water budget, because the Natural Park does not have a complete gauging network. The Basin Júcar Authority only can estimate indirect sources analyzed through management models and the Drainage Board JDA does not make available information on water levels and water pumped to the sea. Moreover, these allocations are below the estimations of the necessities of the lagoon, which have been calculated to be 265 hm^3 per year (Soria & Vicente, 2002). The lack institutional engagement for an ecological flow is regularly discussed in interviews: "*Who decides how much water is allocated for the Albufera? The basin plan does not specify that. Someone needs to issue a mandate for that. We need to do it*" (Interview 2014-6, see supplementary information at http://www.limnetica.net/en/limnetica)[7].

According to the Water Framework Directive, the Albufera is an obligatory restriction for the whole Júcar system, but in practice, it is still the last in the queue, and the agricultural demands are given priority. The environmental water quality objectives established by the WFD for the Albufera do not have to be fulfilled until 2027. Postponing these objectives could be realistic, but it also bestows certain flexibility to the administration to manage the system, and to preserve the priority of the farming sector of the whole Júcar basin, which suffers from enormous political pressure.

The current deficit of the Júcar basin could increase in the next decades due to climate change processes. The Júcar Basin Authority has estimated a 12 % decrease in water resource availability in the whole Júcar basin, whereas other possible scenarios (Marcos-García *et al.*, 2017) have calculated higher decreases in rainfall and water resource availability for 2030. In this possible context, how could the necessities of the Albufera be guaranteed? Will it be possible to maintain the freshwater stage? Some authors have detected significant soil salinity at the rice paddies, and have identified a subsurface flow from the Mediterranean Sea that varies depending on the irrigation practices. This flow produces a salinization of soil horizons in depth, and it is weaker in the southern area, because of the river Júcar water (Moreno-Ramón, 2013; Moreno-Ramón *et al.*, 2015). Moreover, some conservationists link the recent increase of flamingo populations as a possible sign of rising salinity. Are the *Albufera Sostenible* (2004) objectives really possible or even desirable under these dramatically different conditions? Or should ecosystem management start a process of adaptation to a new scenario of salinity in a few years? And finally, as Moreno-Ramón (2013) suggested: Are the *tancats* still sustainable or will renaturalization be required to control the increasing salinity?

BUILDING CONFIDENCE FOR SHARED GOVERNANCE

Since the creation of the Natural Park, social consensus about protection has significantly increased. Protection and natural heritage are now linked: the people of Valencia cherish the wetland, its Devesa and its lagoon, as a protected area. Citizens appear to be more involved, with an increasing number of visitors and local participation in cultural and educational activities. There are new organizations and companies working in environmental tourism (especially birdwatching), foundations such as *Assut* (since 2010) developing awareness of the wetland values through dissemination programs and NGOs such as SEO-Birdlife, or Global Nature and *Acció Ecologista-Agró*, both taking care of the artificial wetlands of *Mília* and *La Pipa* respectively. Other new associations such as *Xuquer Viu* (created in 2003) are committed to solving water quantity problems, promoting a living Júcar river at the basin scale for ecosystems and humans, with fresh flowing water. Two Life programs, fully or partly dedicated to environmental education, have been very successful in recent years –"Environmental Seduction" and "Life Albufera"– facilitating mass visits to the Devesa and to the *La Pipa* green filter.

During the last decade, a number of areas of debate have arisen, drawing attention to the valuable social capital of the Albufera. A citizens' association "*Fira de l'Albufera*" was created in 2011 in order to organize an annual meeting for associations, institutions and inhabitants of the Albufera. This association was configured as a tool for social and cultural dynamization and also as a forum for informal discussion among stakeholders. Six *Fira de l'Albufera de Valencia* events were organized between 2011 and 2016, but unfortunately none in 2017.

Despite this growing social interest and involvement, the park's management lacks of social and political debate across many aspects of decision-making. There is an assembly with a consultative role only, the *Junta Rectora*, but its meetings are scarce. In order to break this isolation, the *Junta Rectora*, in cooperation with the Universitat de València organized an open Simposium in 2016, "*L'Albufera, Ara*", followed by a public exhibition on the history of the civil fight to recover the Devesa.

During this symposium, water management was the most controversial issue. It became evident during the meeting that there was complete lack of a common route sheet for water management, neither for future needs nor for day-by-day practices. Currently, the main stakeholders (River Basin Authority, Autonomous Community with the three authorities concerned, Valencia City Council, researchers from Life Albufera) are working on a new Special Plan, centered on water management. They have begun to meet regularly but there are no concrete results for the moment, and no public dissemination of the debates and progress.

CONCLUSIONS

The most exceptional value of the Albufera is not the internationally recognized birdlife, but its unique water system with its complex mechanisms and its specific set of agro-ecosystems, which is worth conserving and making sustainable. This heritage dimension, thanks to the attachment of the people of Valencia to their Albufera, is the most significant lever for achieving greater sustainability, but also a demanding challenge for managers.

The Natural Park was created to increase sustainability and conservation of the ecosystem. However, management has been marred by unsustainable dynamics and most of all political, institutional and financial inertias (Jégou, 2015). The resulting jigsaw of jurisdictions and stakeholders has dramatically hindered transparency and decision sharing. The key goals of water management and governance principles are unclear and not common to all stakeholders. One of the most evident examples of this lack of transparency and decision sharing is the determinant role still played by the Drainage Board.

Despite this inconsistent institutional context, the lagoon and its Devesa have undergone significant improvements during the last decades. The socio-ecological system should achieve higher sustainability through the implementation of transparency, conciliation and shared actions of water management. Transparency can describe the governance as well as the water quality, as if water quality reflects governance quality. If the governance remains murky, so does the water. This interpretation stems from the "corps médial" of Augustin Berque, the ecosystem being a representation of society, its mesological body (Berque, 1996).

Transparency, conciliation and shared governance require the reformulation of the institutional architecture of the wetland. The recent experience shows that the formula of the Natural Park has been useful to improve wetland conservation and increase environmental awareness, but is insufficient to manage the whole SES, in which other institutions are involved. The integration of all the institutions managing the Albufera through a transversal coordination office is recommended, to move beyond the current fragmentation.

Moreover, mediation is absolutely necessary. Any mediation has remained informal until now. The creation of a permanent observatory, based on the emerging social capital of the wetland, could be the basis with which to strengthen the approach between the two frames –conservationist and productivist - traditionally opposed in the wetland.

This is particularly significant in the current and uncertain environmental context, defined by highly demanding scenarios of climate change and lack of fluvial resources. The threat of increasing salinity could bring into question the mentioned goal of bringing back the Albufera to the idyllic 1960 stage. Most likely, according to the current water resource availability, the challenge is to find the best way to adapt the ecosystem (with the highest water quality levels) to a new hydro-social scenario, instead forcing the system back in time to a past *médiance*.

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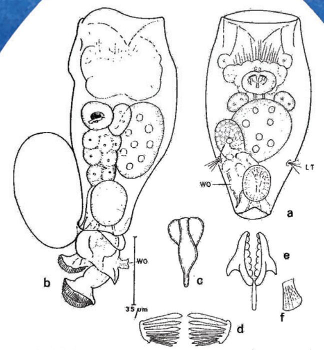

Fig. 2. (a) *Anuraeopsis miraclei*, contracted, ventral; (b) stretched, lateral, Weber's organ length 35 µm; (c)–(f) trophi elements: (c) manubrium, (d) unci, (e) incus, (f) fulcrum.

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