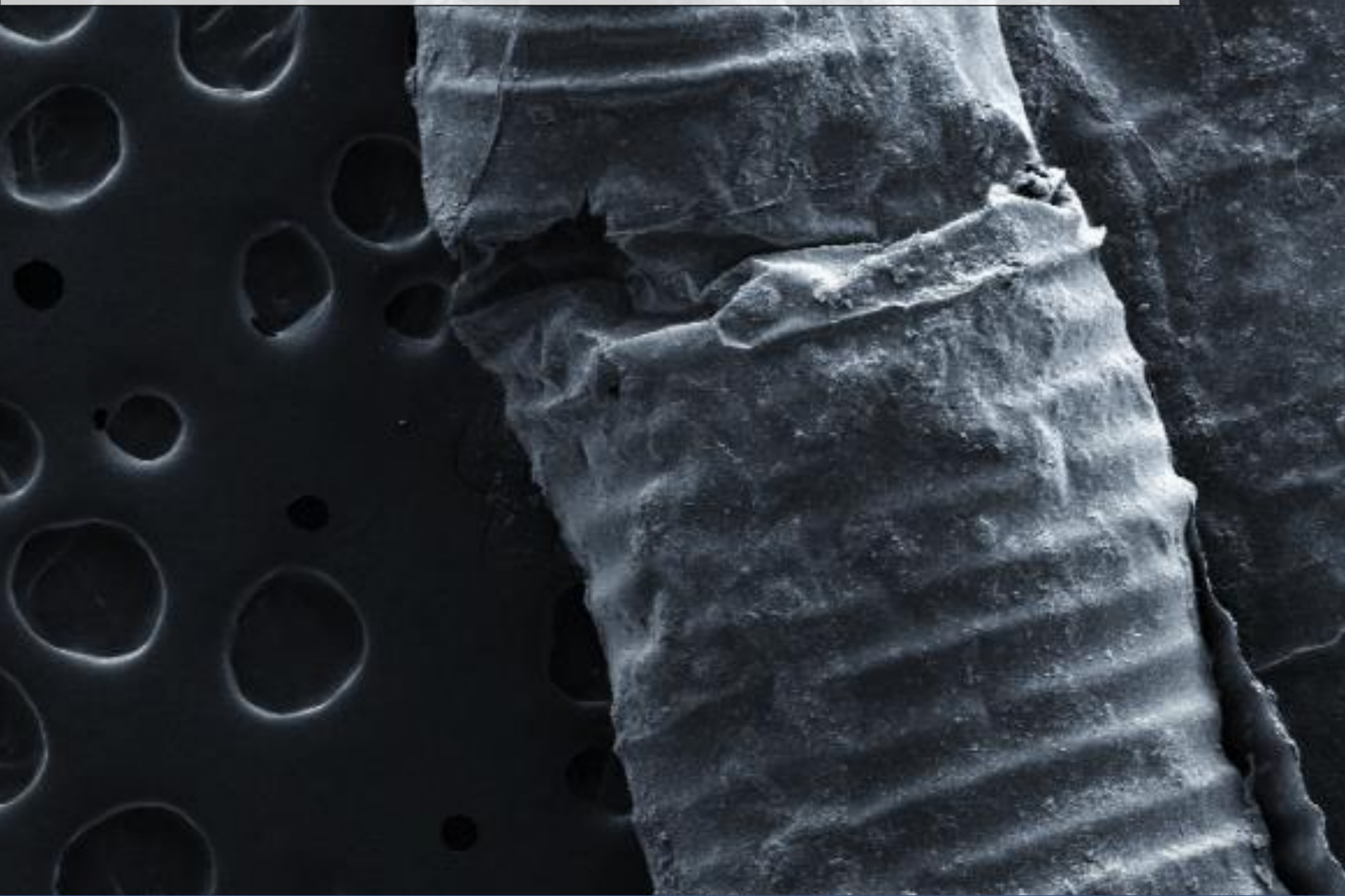
ASSESSMENT OF MICROPLASTICS AND OTHER CONTAMINANTS IN MARINE VERTEBRATES FROM THE WESTERN MEDITERRANEAN SEA

Tesis doctoral por: **Olga Novillo Sanjuan**

Directores: Jesús Tomás Aguirre y Juan Antonio Raga Esteve

València, enero de 2023



PROGRAMA DE DOCTORAT EN BIODIVERSITAT I BIOLOGIA EVOLUTIVA 3101

Facultat de Ciències Biològiques

Universitat de València

ASSESSMENT OF MICROPLASTICS AND OTHER CONTAMINANTS IN MARINE VERTEBRATES FROM THE WESTERN MEDITERRANEAN SEA

Tesis Doctoral por:

Olga Novillo Sanjuan

Directores:

Jesús Tomás Aguirre

Juan Antonio Raga Esteve

València, gener de 2022

VNIVERSITAT DE VALÈNCIA

Facultat de Ciències Biològiques

PROGRAMA DE DOCTORAT
EN BIODIVERSITAT I
BIOLOGIA EVOLUTIVA 3101

VNIVERSITAT DE VALÈNCIA

D. Juan Antonio Raga Esteve, Catedrático de Universidad del Departamento de Zoología de la Facultad de Ciencias Biológicas de la Universitat de València, y D. Jesús Tomás Aguirre, Profesor Contratado Doctor del Departamento de Zoología de la Facultad de Ciencias Biológicas de la Universitat de València,

CERTIFICAN que Olga Novillo Sanjuan ha realizado bajo nuestra dirección, y con el mayor aprovechamiento, el trabajo de investigación recogido en esta memoria, y que lleva por título "*Assessment of microplastics and other contaminants in marine vertebrates from the Western Mediterranean Sea*", para optar al grado de Doctora en Ciencias Biológicas.

Y para que así conste, en cumplimiento de la legislación vigente, expedimos el presente certificado en València, a 26 de enero de 2023.

A mis padres,

a mi abuela,

a mis amigas.

AGRADECIMIENTOS

En primer lugar, me gustaría agradecerles a mis directores Toni y Jesús, el abrirme las puertas del laboratorio y de la investigación. También me gustaría incluir aquí al que fue mi director de Trabajo de Fin de Máster, Pepe Pertusa, por transmitirme ese entusiasmo y energía tan genuina y por estar siempre dispuesto a aconsejarme. Gracias a todos ellos, que confiaron en mi y permitieron que esta tesis pudiera llevarse a cabo.

Por supuesto, querría agradecer también a todo el equipo de la Unidad de Zoología Marina todo su trabajo, dedicación y ayuda. Gracias Patri por enseñarnos tantas cosas, por incluirme en los censos y enseñarme todos los protocolos, por tu bondad y, como no, por ser compañera del café de filtro. Algún día conseguiré que el laboratorio deje las cápsulas jeje. Quería agradecer a Isa, por su infatigable logística y por conseguir todas las cosas raras que le pedía. Pero, sobre todo, por ayudarme tantísimo con la gymkhana que fue enviar las muestras a Noruega. Sin ti, no hubiera conseguido realizar el estudio que más me importaba de la tesis.

Como no, gracias Rachel y Sofía, por ser mis lab-wives y amigas. Por todas las risas, por las tardes de escalada, por todos los momentos. Gracias también a Jaime, por tu compañía, por tu manera de ver y hacer. Gracias Marta por ser fan de mi serie baño-selfie y por esa alegría desbordante tan tuya. Cuando me expongan los selfies en el MoMA te dedicaré la exposición. También a Álex, por compartir frikismo fotográfico, a Paco por estar siempre ahí para una buena charla, a Mar, Natalia, Ana, Aigües, Ohiana, Maria, Jose, David, Javi, y a todas las nuevas incorporaciones por darle vidilla al día a día: Alicia, Mar Izquierdo, Greta. ¡Gracias por esos empujones finales! Gracias también y en especial a Merche, por su energía y por su forma de ver la vida, gracias por todo lo que nos has enseñado. El vino de después del depósito va en tu honor.

I would also like to thank all the colleagues and friends I met at the NTNU in Trondheim. Specially to Veerle Jaspers, who opened their doors for me again and to Alex Asimakopoulos, who kindly hosted me in his lab and let an intruder biologist to enter in their clean chemistry world. I have no space in this acknowledgments to thank you enough for all the help, the learning and the patience I got from you. I'd like to thank the Norwegian-Spanish team too. Ya tiene tela irte al extranjero para hablar inglés y que todos seáis de aquí. Patri, Gabi, Fer, Susana, Dani, Stefano, Martina (os incluyo como buenos Mediterráneos), gracias por todos los momentos dentro y fuera de la universidad. Gracias a todos por acogerme sin miramientos y aportar tanta calidez en un lugar post-pandémico tan frío.

Gracias también a todas las organizaciones que han confiado en mí y han contado conmigo en su lucha medioambiental, especialmente a toda la familia de Xaloc (Juan, Carla, Álex, Carles), a Emilio Beladiez y a sus Bioagradables, a la familia de Ambiens. Gracias por sacarme del laboratorio y por llevar la ciencia a dónde importa.

Estos agradecimientos no estarían completos si no mencionara a mis amigas y a mi familia. Gracias por estar, esta tesis también es vuestra. Masha, Laura y Paquito, estoy muy feliz de conservar nuestra amistad y de conservar la costumbre de los cafés-express electrónicos, aunque aquí siempre falte Paco (ejem). A Elena, porque la quiero como a mi hermana, porque me hace muy feliz crecer juntas y tenerte a mi lado. A Joana, porque conocerte es de lo mejor que me ha pasado. La vida es muy irónica, a veces, pero menos mal. A Alba, Patri y Alejandra, por estar siempre dispuestas a un café y a arreglar el mundo (a.k.a. rajar) en una tarde. A Neus, porque ha sido un pilar esencial en estos últimos años y me ha hecho aprender infinito sobre lo que importa. A Dani, por las risas y por los sueños compartidos de aventuras alpinistas. Gracias por enseñarme a cómo no matarme en una cornisa de nieve. Bueno a ver, en realidad, gracias por el queso.

Por último, gracias a mis padres y a mi abuela, por estar ahí siempre, por ser casa. Iaia, gracias por preocuparte siempre por mi alimentación. Te juro que como bien cuando no estoy en casa. Os quiero mucho.

Agradecimientos Institucionales

Esta tesis ha sido posible gracias a la concesión de una beca predoctoral de Formación de Profesorado Universitario (FPU2017), concedida por el Ministerio de Educación del Gobierno de España.

Los estudios han sido llevados a cabo en el marco de los proyectos europeos INDICIT II (Indicator Impact Taxa nº110661/2018/794561/SUB/ ENV.C2) y MEDSEALITTER (Interreg Mediterranean), así como en el marco del proyecto AICO/2021/022 concedido por la Conselleria d'Innovació, Universitats, Ciència i Societat

Digital, Generalitat Valenciana, y por la Fundación Biodiversidad del Ministerio para la Tranición Ecológica y el Reto Demográfico (MITECO) del Gobierno de España.

Los análisis de esta tesis también se han beneficiado de la financiación aportada por el Departamento de Química de la Universidad Noruega de Ciencia y Tecnología (NTNU, Trondheim, Noruega) y de la Asociación Chelonia gracias al proyecto “Mares Circulares”.

Finalmente, todas las muestras han sido conseguidas gracias a la Red de Varamientos de Cetáceos y Tortugas Marinas de la Comunidad Valenciana, apoyada por la Conselleria de Agricultura, Medio Ambiente, Cambio Climático y Desarrollo Rural de la Generalitat Valenciana.

INDEX

ABBREVIATIONS

CCL - Curved Carapace Length

EDs - Endocrine disruptors

EEA - European Environmental Agency

EPD – Ethylene Propylene Diene

EPS - Expanded polystyrene

EU - European Union

EVOH – Ethylene Vinyl Alcohol

FT-IR - Fourier transformed infrared spectroscopy

GES - Good Environmental Status

HDPE – High Density Polyethylene

ICP - MS - Inductively Coupled Mass Spectrometry

LDPE - Low Density Polyethylene

MP - Microplastic

MSFD - Marine strategy framework directive

PAEs - Phthalate esters

PBT - Polybutyl Terephthalate

PCPs - Personal Care Products

PE – Polyethylene

PIB – Polyisobutylene

PMMA - Polymethyl Methacrylate

POPs - Persistent Organic Pollutants

PP – Polypropylene

PUR – Polyurethane

PVF – Polyvinyl Fluoride

REACH - European Regulation on Registration, Evaluation, Authorisation and Restriction of Chemicals

SEM - Scanning Electron Microscopy

SDG - Sustainable Development Goals

SPE - Solid Phase Extraction

UHPLC-MS - Ultra-high performance liquid chromatography - Mass Spectrometry

UNEP - United Nations Environment Programme

WWTP - Wastewater treatment plant

Phthalate metabolites:

mEP - Monoethyl phthalate

mBP - Mono-n-butyl phthalate

mIBP - Mono-iso-butyl phthalate

mPeP - Mono-n-pentyl phthalate

mIPeP - Mono-iso-pentyl phthalate

mHxP - Mono-n-hexyl phthalate

mHepP - Mono-n-heptyl phthalate

mCHP - Monocyclohexyl phthalate

mBzP - Monobenzyl phthalate

mOP - Mono-n-octyl phthalate

mCPP - Mono(3-carboxypropyl) phthalate

mNP - Mono-n-nonyl phthalate

mDeP - Mono-n-decyl phthalate

mEHP - Mono(2-ethyl-1-hexyl) phthalate

mEOHP - Mono(2-ethyl-5-oxohexyl) phthalate

mEHHP - Mono(2-ethyl-5-hydroxyhexyl) phthalate

PA - Phthalic acid

SUMMARY

Marine ecosystems are under great pressure due to climate change, overfishing, and other anthropogenic activities; producing waste that pollutes water masses with new materials and products produced to satisfy consumer needs. The sources, transport, fate, degradation and impact of these pollutants are not fully understood; therefore, we are in need of increasing knowledge about their behaviour and potential effects in the marine environment.

The Mediterranean Sea is especially vulnerable to pollution due to its closed nature. Water exchange with the Atlantic Ocean is limited and it is even more restricted with the Red Sea, fact that makes it prone to accumulate more contaminants than in the ocean. This situation puts marine biodiversity under threat. Specially in the case of non-commercial species, the presence of pollutants and their effects are not well studied due to their lack of direct economic value or their non-consumption by humans. For this reason, the main objective of this thesis was to analyse the presence of a series of pollutants of concern in non-commercial species in order to know their exposure levels and establish baseline values.

The studies carried out along this thesis have been performed in the western part of the Mediterranean Sea (Valencian Community, East Spain), where we can easily find loggerhead turtles (*Caretta caretta,* Linnaeus, 1758), striped dolphins (*Stenella coeruleoalba,* Meyen 1833), and jewel lanternfish (*Lampanyctus crocodilus,* Risso 1810), which is described as important prey for the mentioned cetacean species. These three species could be exposed to important amounts of organic chemicals, heavy metals and plastic waste in this area; regarding that, the near land (East Spain) is a known source of multiple contaminants due to intensive agriculture, mass tourism, high urbanization and population concentrations. Regarding this situation, in this thesis we analysed the exposure of the mentioned species to different pollutants, establishing baselines for future monitoring studies and providing data for potential mitigation plans.

In particular, in this thesis, we:

1. Analysed pesticides, heavy metals and phthalates in tissues of freshly dead loggerhead turtles by using chromatographic and spectrometric techniques. UHPLC-MS/MS was used to perform a non-targeted screening to analyse pesticide exposure in muscle

and fat tissue, ICP-MS was used to analyse Cd, Hg and Pb concentration in muscle and fat tissues, and, lastly, another UHPLS-MS/MS was used to analyse the concentration of phthalate metabolites, kown as common plasticizers, in turtles' livers.

2. Quantified and determined the polymers of microplastics and microdebris present in beaches of the Valencia Province, which are sporadically used as nesting grounds by loggerhead turtles currently in the Spanish Mediterranean coasts. We aimed to see whether microplastics could pose a threat to loggerheads' nesting success and whether factors such as anthropic use and time of the year could influence the amount of microdebris found. For this purpose, sand was collected and separated through a series of density separations. The synthetic items found were subsequently analysed by FT-IR to obtain their polymeric composition.

3. Quantified striped dolphins' exposure to microplastics through the analyses of their digestive contents, testing their role as bioindicators of microplastic pollution at sea. For this purpose, a basic digestion of the contents was performed, followed by filtrations under vacuum. Lastly, the collected items were analysed in order to know their polymeric composition.

4. Quantified jewel lanternfish exposure to microplastics through the analyses of their digestive contents and tested their role as bioindicators of microplastic pollution in the bathypelagic environment. Microplastics were separated through basic digestion with KOH 10% and observed under stereomicroscope.

Results in this thesis indicate the ubiquity of all the analysed pollutants, although with differences in exposure among species and habitats. Next, we present the general results and conclusions:

Samples for pesticide analyses showed that all the loggerhead turtles analysed (N = 25) were exposed to 39 different pesticides, of which 38.5% are forbidden in the European Union. Concerning pesticide use, most of them were insecticides (12), followed by fungicides (11), herbicides (9), acaricides (3), and a rodenticide. The non-approved pesticides could have been incorporated to body tissues before arriving to EU waters, while migrating through non-EU Mediterranean countries in which their use is allowed, or in Mediterranean countries where, although forbidden, they are still used. In this study, body size or stranding location of the turtles did not influence pollutant burden, meaning that loggerheads in this study are exposed equally.

Results indicated generally low concentrations of heavy metals in contrast to studies carried out in the eastern Mediterranean, possibly because lower water circulation in the East, which tends to concentrate pollutants. Specifically, mean concentrations of Cd, Pb and Hg were 0.04 μg/g w.w., 0.09 μg/g w.w. and 0.03 μg/g w.w. in fat and 0.05 μg/g, 0.08 μg/g and 0.04 μg/g in muscle, respectively. Juvenile size and developmental stage of turtles analysed could account for these low concentrations. The results of this study showed that size and metal concentration in tissues were not correlated, neither in fat nor in muscle (Spearman correlation test, $p > 0.05$ in all cases). Geographically, metal concentrations in turtles found North and South de la Nao Cape were not significantly different (U Mann-Whitney, $p > 0.005$).

For phthalate metabolite analyses, loggerhead turtles' livers were extracted by SPE and analysed by UHPLC-MS/MS. Seven of the eighteen analysed metabolites, including monomethyl phthalate (mMP), phthalic acid (PA), monoethyl phthalate (mEP), mono-n-butyl phthalate (mBP), mono-n-hexyl phthalate (mHxP), mono-n-nonyl phthalate (mNP), and mono-n-heptyl phthalate (mHepP) were detected in more than 85% of the samples (DR>85%). The highest medians were showed mono-n-decyl phthalate [mDeP (38.9 ng/g d.w.)] followed by PA (24.2), mono(2-ehyl-1-hexyl) phthalate [mEHP (22.2)], and mHxP (20.2 ng/g d.w.). The sum of the median concentration of the phthalate metabolites that had DRs above 85% (12 metabolites), showed a slight negative correlation with size of the turtles. This sum of the medians also showed a significant increase in phthalate metabolites concentrations from 2020 onwards ($p = 0.0005$), possibly explained by an increase in single-use plastic and its mismanagement following the Sars-Cov-2 pandemic, or by uncontrolled wastewater discharges in the sea. These results show a high prevalence of plasticizers in loggerhead turtles' livers, indirectly supporting the exposure to plastics and other products where they are present, in the Mediterranean Sea.

Microdebris in potential nesting beaches was ubiquitous although concentrations were not of concern for the hatching success and embryo development of loggerhead turtle clutches. Results indicated that in July total mean±SD was 5.66 ± 3.66 MPs/kg at surface and 12.15 ± 7.76 MPs/kg at depth; while in November values were 6.45 ± 4.42 MPs/kg at surface and 5.51 ± 3.14 MPs/kg at depth. There were no significant differences among beaches, months, depths nor protection regime of the beach; hence, level of isolation/use of the beach could not be used as a predictor in this case. Polymers found were, by descent order, polyethylene, rubber, latex, polypropylene and ethylene vinyl alcohol; which are of common use in consumer goods, tires and food packaging. On the other hand, macroplastics were abundant and concerning at the sampling sites. Therefore, it is essential to continue monitoring these beaches, regarding that microdebris can be originated from these big items.

Striped dolphins in this study were not at risk by the ingestion of microplastics, although it is noteworthy that this contaminant was present in almost all of them. After the basic digestion and filtration of 43 striped dolphins' digestive systems, 90.5% of them contained microplastics. Of these microplastics, 73.6% were fibres, 23.87% were fragments and 2.53% were pellets. Despite the high frequency of occurrence, microplastic amount per dolphin was relatively low and highly variable (mean ± SD = 14.9 ± 22.3; 95% CI: 9.58–23.4; median = 5, range = 0 - 82). Through FT-IR spectrometry, we found that polyacrylamide (40.9%), typically found in synthetic clothes, was the most common plastic polymer, followed by PET (27.3%), and HDPE (9.1%). In total, 73% of the polymers were synthetic, and the rest (15.4%) were not plastics (alginic acid and cellulose). Microplastic concentration in dolphins from different periods of time (1989 - 2007 and 2010–2017) or between locations did not show significant differences. With regards to their use as microplastics bioindicator, other species appear to be more adequate for this purpose.

Finally, the jewel lanternfish, basic digestion and filtration of their digestive contents (N = 97), revealed high frequency of occurrence of microfibres, although a low body burden. Almost half (40.21%) of the analysed jewel lanternfish had microplastics, mostly blue and black fibres (40.9% and 34.66%, respectively), as it is common in other studies, including in striped dolphins analysed in this thesis. In total, 185 microplastics (97.75 % fibres) items were identified. In fishes with at least one microplastic, the median was 3 MPs/fish (CI 95% = 3.46 – 6.8); similar to other studies performed in other fish species in the area. Biometric parameters, including total length and body condition (Fulton's K), were not correlated with the number of microplastics.

To sum up, in this thesis, an overview of the variety of pollutants in the westernmost part of the Mediterranean Sea is provided, focusing on the aforementioned species and highlighting their potential as bioindicators of the environmental health of the Mediterranean Sea.

RESUMEN

Los ecosistemas marinos están bajo una gran presión debido al cambio climático, la sobrepesca y otras actividades antropogénicas; produciendo residuos que contaminan las masas de agua con nuevos materiales y productos elaborados para satisfacer las necesidades de los consumidores (O'Hara and Halpern, 2022). Las fuentes, el transporte, el destino, la degradación y el impacto de estos contaminantes todavía no se entienden por completo; por lo tanto, nos vemos en la necesidad de aumentar el conocimiento sobre su comportamiento y efectos potenciales en el medio marino.

El mar Mediterráneo es especialmente vulnerable a la contaminación debido a su carácter cerrado. El intercambio de agua con el Océano Atlántico es limitado y está aún más restringido con el Mar Rojo, hecho que lo hace propenso a acumular más contaminantes que en aguas más abiertas. Los contaminantes lixiviados al medio ambiente no solo permanecen en matrices abióticas, sino que también se incorporan a la biota a través de diferentes vías. Esta situación pone en riesgo la biodiversidad marina. Especialmente en el caso de especies no comerciales, la presencia de contaminantes y sus efectos no están bien estudiados debido a su falta de valor económico directo o su no-consumo por parte de los humanos. Por ello, el objetivo principal de esta tesis es analizar la presencia de una serie de contaminantes de interés en especies no comerciales y así conocer sus niveles de exposición y establecer valores de referencia que sean de utilidad para evaluar la calidad ambiental del mar Mediterráneo.

Los estudios realizados a lo largo de esta tesis se han realizado en la parte occidental del Mar Mediterráneo (Comunidad Valenciana, Este de España), donde podemos encontrar fácilmente tortugas bobas (*Caretta caretta*, Linnaeus, 1758), delfines listados *(Stenella coeruleoalba*, Meyen 1833), y peces linterna (*Lampanyctus crocodilus*, Risso 1810), el cual se describe como presa importante para las especies de cetáceos mencionadas. Estas tres especies han sido propuestas como organismos indicadores de la salud ambiental del Mediterráneo. Además, podrían estar expuestas a cantidades importantes de químicos orgánicos, metales pesados y plásticos en esta zona, ya que la costa próxima (Este de España) es una fuente conocida de múltiples contaminantes debido a la agricultura intensiva, el turismo de masas, la excesiva urbanización y la abundante población (Moreno-González et al. 2013; UNEP/MAP and Plan Bleu, 2020). En este escenario de múltiples estresores, la conservación y cuidado de los océanos se ha convertido en una de las mayores prioridades de los Objetivos del Desarrollo Sostenible de las Naciones Unidas (ODS, Naciones Unidas

2030) y muchos esfuerzos se han centrado en construir herramientas legislativas en la Unión Europea (UE) como la Convención de Barcelona para la protección del Mar Mediterráneo (Alava et al. 2020) y la creación de la directiva *Marine Strategy Framework Directive* 2008/56/EC (MSFD). Teniendo en cuenta todos estos factores, en esta tesis analizamos la exposición de las especies mencionadas a diferentes contaminantes, estableciendo líneas de base para futuros estudios de monitorización y brindando datos para potenciales planes de mitigación. Los contaminantes aquí estudiados son, en orden de aparición: pesticidas, metales pesados, metabolitos de ftalatos y microplásticos.

Los pesticidas son compuestos químicos utilizados para el control de plagas en diferentes ámbitos, pero sobretodo en la agricultura. Pueden ser persistentes y no persistentes. Los primeros se metabolizan muy lentamente y como consecuencia pueden permanecer en el medio ambiente durante largos períodos de tiempo, acumulándose y provocando efectos tóxicos (Pérez-Lucas et al. 2019). De los pesticidas no persistentes, se sabe relativamente poco, ya que se consideran seguros para los organismos que no son su diana, aunque faltan datos sobre su presencia y efectos en numerosas especies (Fernández et al. 2020; Yusà et al. 2022).

Los ftalatos son aditivos que se le añaden a los plásticos para darles cualidades concretas como elasticidad o durabilidad, aunque también se utilizan en otros productos como en perfumes, herbicidas y en otras aplicaciones industriales. Los ftalatos no están unidos de manera covalente a los polímeros principales, de manera que se desprenden fácilmente y pueden ser incorporados por los seres vivos rápidamente, donde son metabolizados (Net et al. 2015; Hu et al. 2016; Asimakopoulos et al. 2016; Rian et al. 2020). Tanto algunos pesticidas como los ftalatos, son considerados disruptores endocrinos (DEs) con el potencial de modificar la respuesta hormonal y de provocar, entre otros efectos, sesgos en la proporción de hembras y machos en una población determinada y disminución de la calidad del esperma (Hlisníková et al. 2020). No obstante, los estudios sobre estos efectos en especies silvestres son escasos. Además, en el caso de los ftalatos, los estudios son complicados ya que la contaminación externa es muy difícil de controlar, dada la ubicuidad de estas especies químicas en el ambiente.

Los metales pesados en el Mediterráneo pueden tener origen natural por la propia composición de los sedimentos o provenientes de la deposición atmosférica, pero también pueden tener origen antrópico (Grousset et al. 1995; Guerzoni et al. 1999). En este caso, son considerados contaminantes heredados y bien estudiados. No obstante, dada su naturaleza persistente y potencialmente tóxica, es de interés continuar monitorizando los sus niveles. En el mar Mediterráneo ha sido común la contaminación por metales debido al pasado minero de algunas de sus regiones (Grousset et al. 1995), con lo cual, es de esperar que algunos de ellos sigan apareciendo en los análisis. Muchos metales están asociados a graves problemas neurotóxicos y genotóxicos, entre otros, de manera que también pueden suponer un peligro para los seres vivos marinos si estos se encuentran en su medio.

Por otra parte, los microplásticos son una de las mayores preocupaciones en cuanto a contaminación en la actualidad. Actualmente, ya se sabe que se distribuyen en todos los medios estudiados, desde las áreas costeras hasta en las profundas fosas oceánicas. Los microplásticos son plástico de un tamaño no mayor de cinco milímetros según la definición de la GESAMP (*Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection*) en su dimensión más grande, y pueden ser primarios o secundarios dependiendo de su origen. En el caso de que los microplásticos sean primarios, habrán sido producidos directamente con ese tamaño o bien para después fundirlos y obtener piezas más grandes, o bien para aplicaciones industriales y como erosivos. Los microplásticos secundarios, por otra parte, son aquellos originados a partir de objetos de plástico más grandes debido al su desgaste y su rotura. Estos microplásticos pueden bloquear el tracto intestinal de organismos pequeños, induciendo a una posible desnutrición (Sëtala et al. 2014) y pueden llevar adheridos otros contaminantes químicos presentes en el medio y actuar como vectores hacia los organismos que los ingieren, ya sea de manera accidental o intencionada (Cole et al. 2011; Hidalgo-Ruz et al. 2012; Andrady, 2017).

En particular, en esta tesis:

1. Se analizan pesticidas, metales pesados y ftalatos en tejidos de tortugas bobas (*C. caretta*) recién muertas mediante técnicas de separación e identificación cromatográficas y espectrométricas. Las tortugas bobas se consideran buenas como especie centinela de la salud ambiental de los mares debido a su largo ciclo de vida, ecología trófica, uso del hábitat y naturaleza migratoria. Especialmente su dieta oportunista y conocida ingesta de basura marina, las hace un buen reflejo de la contaminación que la rodea. Para el análisis de compuestos orgánicos se realizó una extracción de los tejidos de grasa y músculo con metanol, así como una posterior filtración con filtros de teflón. Se utilizó cromatografía líquida UHPLC-MS/MS TOF (*Ultra High Performance Liquid Chromatography – Mass Spectrometry Time of Flight*) para realizar un cribado no dirigido y analizar la presencia de pesticidas en tejido muscular

y graso. Este análisis fue posible gracias a la comparación de los espectros obtenidos con una base de datos previa que incluía pesticidas ya encontrados en esta área en otras ocasiones. Para analizar la concentración de cadmio (Cd), mercurio (Hg) y plomo (Pb) en esos mismos tejidos, se realizó na digestión ácida con ácido nítrico (HNO_3) y una posterior filtración. Posteriormente se utilizó espectrometría ICP-MS (*Inductively Coupled Mass Spectrometry*) que permitió determinar la concentración exacta de los metales. Por último, se realizó una extracción de fase sólida (SPE, *Solid Phase Extraction*) para poder cuantificar metabolitos de ftalatos en hígados de las tortugas bobas. Los metabolitos de ftalatos son producto del metabolismo de aditivos plásticos e industriales, que nos indica el contacto de los organismos con estos compuestos en principio ajenos al medio natural. Tras la extracción de fase sólida se procedió a la evaporación de las muestras bajo un flujo de nitrógeno y a su posterior reconstitución con acetonitrilo, para poder hacer, finalmente, una cromatografía UHPLC- MS/MS que indicó la concentración de metabolitos de ftalato en los hígados analizados. La identificación de los compuestos se realizó gracias al método de los estándares internos. En este método, se le añade a las muestras una cantidad conocida del compuesto estudiado, permitiendo así la posterior comparación y cuantificación de los compuestos problema.

2. Se cuantifican y determinan los polímeros de microplásticos y microbasura presentes en tres playas de la provincia de Valencia. Las tres playas estudiadas son utilizadas esporádicamente como playas de nidificación por las tortugas bobas del Mediterráneo occidental. El objetivo principal de esta investigación fue evaluar si los microplásticos podrían representar una amenaza para el éxito de anidación y desarrollo embrionario de las tortugas bobas, y si factores como el uso antrópico y la época del año podrían influir en la cantidad de microbasura encontrada. Por ello, se eligió una playa urbana (playa del Cabanyal) con gran afluencia de visitantes y, por lo tanto, sometida a una mayor presión antropogénica. Por otra parte, se eligieron dos playas dentro del parque natural de l'Albufera. Una de ellas, situada justo delante de un hotel de lujo y con acceso restringido (playa de l'Alcatí); la otra, localizada a continuación, pero de acceso prohibido (playa de la Punta), es decir, con una presión antropogénica de mucha menor magnitud. Además, estas dos últimas playas están localizadas lejos del núcleo urbano de la ciudad de Valencia y son, por tanto, menos frecuentadas debido a la lejanía y al transporte público escaso. Para separar la

microbasura de la arena de las playas, la arena fue filtrada varias veces consecutivas. Primero, con un cedazo de luz de malla de 5mm, que permite descartar la fracción macro de los plásticos. Una vez en el laboratorio, la arena previamente separada de la fracción macro, fue mezclada con solución salina (NaCl) supersaturada para realizar una separación por densidad. Esta separación por densidad permite que los microplásticos floten debido a su menor densidad. Los elementos sintéticos encontrados fueron separados del sobrenadante gracias a una filtración al vacio con filtros de microfibra GF/C. Estos filtros, fueron observados a lupa y los ítems encontrados fueron separados y posteriormente analizados por espectrometría infraroja FT-IR (*Fourier Transformed Infrared Spectrometry*) para obtener su composición polimérica.

3. Se cuantifica la exposición de delfines listados (*S. coeruleoalba*) del Mediterráneo occidental a microplásticos a través del análisis de su contenido digestivo, probando así su posible papel como bioindicadores de contaminación por microplásticos en el medio pelágico. El valor de este estudio radica en la información indirecta que nos proporciona sobre el medio pelágico, no muy estudiado debido a su dificultad de muestreo. Por otra parte, en este estudio se maneja una muestra grande de delfín listado que nos permite tener una evaluación más completa de lo que es habitual, ya que, por motivos de tamaño del animal, tiempo de filtración y dificultad de muestreo, los estudios con cetáceos suelen usar muy pocos ejemplares. Para separar los microplásticos de los contenidos digestivos, se realizó una digestión básica con KOH al 10%. Esta solución se dejó reposar 3 semanas, ya que el contenido estomacal de estos cetáceos es rico en materia orgánica y es muy denso para filtrar. Una vez pasadas las tres semanas, se realizaban una serie de filtraciones al vacío con filtros de microfibra GF/C y un embudo de Büchner. Los filtros resultantes, se secaban durante 24h y se observaban posteriormente a lupa. Por último, se los elementos antropogénicos encontrados o los elementos sospechosos de serlo, se analizaron los para conocer su composición polimérica mediante espectrometría infrarroja FT-IR.

4. Se cuantifica la exposición de peces linterna (*L. crocodilus*) a microplásticos a través del análisis de su contenido digestivo, y se pone a prueba su papel como bioindicadores de la contaminación por microplásticos la zona batipelágica del medio marino. Los peces linterna constituyen una parte vital del medio oceánico, ya que son

una parte importante de la bomba de carbono en los ciclos biogeoquímicos y trasladan materia orgánica a lo largo de la columna de agua gracias a sus migraciones diarias. Así pues, nos pueden ofrecer información indirecta de valor sobre la circulación de los contaminantes en el océano. El contenido digestivo de los ejemplares de *L. crocodilus* utilizados en este estudio se digirieron en KOH al 10 % y posteriormente se filtraron al vacío con filtros de microfibra GF/C y con un embudo de Büchner. Posteriormente, estos filtros se secaron durante 24h en una estufa y se observaron a lupa y a microscopio para determinar su naturaleza artificial.

Los resultados de esta tesis indican la ubicuidad de todos los contaminantes analizados en el Mediterráneo occidental, aunque con diferencias en la exposición entre especies y hábitats. Todas las muestras de las especies que se han utilizado en esta tesis, han sido obtenidas gracias a la Red de Varamientos de Cetáceos y Tortugas Marinas de la Comunidad Valenciana, que permite la rápida actuación en caso de varamiento o captura accidental de especímenes de estos grupos animales. Los individuos frescos obtenidos gracias a eta red, eran rápidamente sometidos a necropsia, lo que permitía el almacenamiento de muestras en estado óptimo o su procesamiento en el instante.

A continuación, presentamos los resultados generales y conclusiones:

Las muestras para análisis de pesticidas mostraron que las tortugas bobas analizadas (N = 25) estuvieron expuestas a una variedad de 39 pesticidas diferentes, de los cuales el 38,5% están prohibidos en la Unión Europea (UE). Hasta la fecha, ningún estudio había realizado esta técnica con tortugas bobas, lo cual permitió determinar la presencia de algunos pesticidas por primera vez en esta especie. En cuanto al uso de pesticidas, la mayoría fueron insecticidas (12), seguidos de fungicidas (11), herbicidas (9), acaricidas (3) y de un rodenticida. Cabe señalar que se detectó la presencia de DDT (dicloro difenil tricloroetano) en una muestra. Este insecticida es altamente tóxico y persistente y por ello está prohibido y contemplado en la Convenio de Estocolmo sobre Contaminantes Orgánicos Persistentes (COPs). Los plaguicidas no aprobados podrían haberse incorporado a los tejidos antes de llegar a aguas de la UE, mientras migraban a través de aguas de países mediterráneos no comunitarios en los que su uso está permitido, o bien mientras migraban por países mediterráneos donde, aunque prohibidos, todavía se utilizan. Excepto por el DDT, el resto de pesticidas encontrados tenían un coeficiente octanol-agua (K_{ow}) bajo, es decir, que su potencial bioacumulación y persistencia es baja. No obstante, el hecho de que aparezcan en las muestras, parece indicar una exposición contínua a estos productos. En este estudio, el tamaño o la ubicación del varamiento de las tortugas no influyó en su carga de contaminantes, lo que significa que las tortugas bobas en este estudio están expuestas a los pesticidas de manera homogénea.

Los resultados del análisis de metales pesados indicaron concentraciones generalmente bajas en contraste con los estudios realizados en el Mediterráneo oriental, posiblemente debido a una menor circulación de agua en el Este, que tiende a concentrar contaminantes. En concreto, las concentraciones medias de Cd, Pb y Hg fueron de 0,04 µg/g peso húmedo (p.h.)., 0,09 µg/g p.h. y 0,03 µg/g p.h. en grasa y 0,05 µg/g p.h., 0,08 µg/g p.h. y 0,04 µg/g p.h. en músculo, respectivamente. El tamaño juvenil y la etapa de desarrollo de las tortugas analizadas podrían explicar estas bajas concentraciones, ya que los metales pesados tienen a aparecer con concentraciones más elevadas en los individuos de más tamaño y más edad. Los resultados de este estudio mostraron que el tamaño y la concentración de metales en los tejidos no estaban correlacionados, ni en la grasa ni en el músculo (prueba de correlación de Spearman, $p > 0,05$ en todos los casos). Geográficamente, las concentraciones de metales en las tortugas encontradas al norte y sur del Cabo de la Nao no fueron significativamente diferentes (U Mann-Whitney, $p > 0,005$).

Para los análisis de metabolitos de ftalatos, se procesaron las muestras de hígado de tortugas bobas mediante extracción de fase sólida y se analizaron mediante cromatografía UHPLC-MS/MS. La extracción de los contaminantes incluyó pasos de sonicación para romper los tejidos y de incubación con ß-glucuronidasa, para desconjungar los compuestos. Siete de los dieciocho metabolitos analizados, incluyendo ftalato de monometilo (mMP), ácido ftálico (PA), ftalato de monoetilo (mEP), ftalato de mono-n-butilo (mBP), ftalato de mono-n-hexilo (mHxP), mono-n- ftalato de nonilo (mNP) y ftalato de mono-n-heptilo (mHepP), se detectaron en más del 85% de las muestras (DR>85%), lo que indica la presencia normal de plastificantes y aditivos industriales en esta especie salvaje y protegida. Las medianas más altas las mostraron los metabolitos ftalato de mono-n-decilo [mDeP (38,9 ng/g peso seco.)] seguido de PA (24,2), ftalato de mono(2-etil-1-hexilo) [mEHP (22,2)] y mHxP (20,2 ng/g p. s.). La suma de la concentración mediana de los metabolitos de ftalatos que tenían tasas de detección superiores al 85%, mostró una ligera correlación negativa entre las concentraciones de metabolitos y el tamaño de las tortugas. Es decir, que cuanto más grande era la tortuga, ligeramente menor era la concentración de metabolitos de ftalatos. Esta suma de medianas también mostró un aumento significativo en las concentraciones de metabolitos de ftalatos a partir del año 2020 ($p = 0,0005$), posiblemente explicado o bien por un aumento del plástico de un solo uso y a su mala gestión tras la pandemia del Sars-Cov-2, o bien por vertidos descontrolados de aguas residuales en el mar. No obstante, en este aspecto, se necesita más información para poder llegar a conclusiones sólidas. Los resultados de este estudio muestran una alta prevalencia de plastificantes en el hígado de las tortugas bobas en el Mediterráneo, lo que indirectamente respalda la idea de que las tortugas bobas del Mediterráneo están frecuentemente expuestas a plásticos y, por tanto, a sus aditivos; entre otros contaminantes.

La microbasura en las posibles playas de anidación fue omnipresente, aunque las concentraciones no parecieron preocupantes para la eclosión y el desarrollo embrionario de los huevos de las tortugas bobas. Los resultados indicaron una media total±DE de 5,66±3,66 MPs/kg en superficie y de 12,15±7,76 MPs/kg en profundidad en el mes de julio; mientras que en noviembre los valores fueron 6,45±4,42 MPs/kg en superficie y 5,51±3,14 MPs/kg en profundidad. No hubo diferencias significativas entre playas, meses, profundidades ni régimen de protección de la playa; por lo tanto, estos factores no pudieron usarse como predictores en este caso. Así pues, el nivel de afluencia y la temporada parecen no afectar a la cantidad de microbasura encontrada en las playas de este estudio. Los polímeros encontrados fueron, por orden descendente: polietileno (PET), caucho, látex, polipropileno (PP) y alcohol etilenvinílico (EVOH); todos de uso común en bienes de consumo, neumáticos y envases de alimentos. Cabe destacar que durante los muestreos de microbasura, grandes cantidades de macrobasura y macroplásticos fueron avistados. Por lo tanto, es fundamental continuar con el monitoreo de estas playas, ya que son estos macroplásticos y grandes desechos los que pueden originar, a su vez, multitud de microbasura, incluyendo microplásticos. Por otra parte, algunos estudios vinculan la acumulación de microplásticos con un incremento de temperatura de la arena de la playa, lo cual podría afectar a la temperatura de incubación de los nidos de las tortugas marinas. Dado que las tortugas son reptiles cuyo sexo es determinado por la temperatura de incubación, es de vital importancia controlar la presencia de contaminantes en este medio. Este fenómeno junto con un incremento generalizado de las temperaturas a causa del calentamiento global, podría dar lugar a una población mayoritariamente de hembras, con el riesgo que ello conlleva del colapso de una población. Así pues, pese a que las cantidades encontradas por este estudio no son muy elevadas, es importante mantener la atención sobre la evolución de esta problemática.

En cuanto a los microplásticos en delfines listados, después de la digestión básica y la filtración de los sistemas digestivos de 43 individuos, el 90,5 % de ellos contenía microplásticos. Es decir, la gran mayoría de individuos había ingerido, al menos, un microplástico, indicando la ubicuidad de este contaminante incluso en depredadores pelágicos. De los microplásticos encontrados, el 73,6% eran fibras, el 23,87% fragmentos y el 2,53% gránulos de origen industrial o *pellets*. A pesar de la alta frecuencia de aparición, la cantidad de microplásticos por delfín fue relativamente baja y muy variable (media±DE = 14,9±22,3; IC del 95 %: 9,58–23,4; mediana = 5, rango = 0 – 82 microplásticos por delfín). A través de la espectrometría infrarroja FT-IR, encontramos que la poliacrilamida (40,9 %), normalmente encontrada en la ropa confeccionada con fibras sintéticas, fue el polímero más común, seguida del PET (27,3 %) y del polietileno de alta densidad (HDPE, 9,1 %). En total, el 73% de los polímeros obtenidos fueron de origen sintético y, el resto (15,4%), no resultaron ser plásticos, si no ácido algínico y celulosa. La concentración de microplásticos en delfines de diferentes períodos de tiempo (1989 - 2007 y 2010-2017) o entre ubicaciones dentro de la Comunidad Valenciana, no mostró diferencias significativas. Los delfines listados de este estudio no estaban en riesgo por la ingestión de microplásticos, aunque es de notar que este contaminante estuviera presente en casi todos ellos. En cuanto al uso de delfines como bioindicador de microplásticos, otras especies parecen más adecuadas. La gran longitud de sus sistemas digestivos y la complejidad de sus contenidos estomacales, hacen muy largo y dificultoso el protocolo de extracción de los microplásticos. Por otra parte, con la información obtenida no podemos concluir si los delfines listados ingieren los microplástico a través de la dieta, por ingestión directa del agua en la que bucean o incluso del aire cuando salen a respirar.

Finalmente, en cuanto a los microplásticos en los peces linterna, la digestión básica y la filtración de su contenido digestivo (N = 97) también mostró una alta frecuencia de aparición de microfibras, aunque una baja cantidad por individuo. Casi la mitad de los peces linterna (40,21 %) analizados tenían microplásticos, en su mayoría fibras azules y negras (40,9 % y 34,66 %, respectivamente). Estos resultados están en línea con otros estudios, incluyendo el estudio de microplásticos en delfines listados analizados en esta tesis. En total, se identificaron 185 microplásticos (97,75 % fibras). En peces con al menos un microplástico, la mediana fue de 3 MP/pez (IC 95% = 3,46 – 6,8); similar a otros estudios realizados en otras especies de peces de la zona. Los parámetros biométricos, incluida la longitud total y la condición corporal (K de Fulton), no se correlacionaron con la cantidad de microplásticos; es decir, tampoco se podía relacionar la salud de los peces con la vulnerabilidad a la ingestión de microplásticos. Finalmente, y teniendo en cuenta que los delfines listados son un depredador natural importante de los peces linterna, sería de interés realizar estudios de transferencia trófica de los microplásticos. La similitud en algunos resultados obtenidos entre este estudio y el de los delfines listados sugiere una potencial transferencia de este contaminante a través de la cadena trófica en esta zona del Mediterráneo ya que, además, los ejemplares de ambos estudios se solapan espacialmente.

En resumen, en esta tesis se proporciona una visión general de la variedad de contaminantes en la parte más occidental del Mar Mediterráneo, centrándose en las tortugas bobas, los delfines listados y los peces linterna. Se proporcionan datos que completan el conocimiento sobre la distribución de contaminantes en especies poco estudiadas en este aspecto, y normalmente difíciles de muestrear. Además, se destaca el potencial de estas especies como bioindicadores de la salud ambiental del Mar Mediterráneo y como método indirecto para obtener información sobre la contaminación del medio marino. Si bien queda clara la ubicuidad de estos contaminantes antrópicos en las especies marinas, quedan por establecer umbrales de toxicidad que permitan determinar en qué medida las cantidades encontradas en estos estudios pudieran ser nocivas para sus individuos y, por tanto, poner en peligro la conservación de las especies. Pese a no encontrar cantidades especialmente preocupantes, todavía sabemos poco sobre los efectos sub-letales que podrían ser resultado de la exposición crónica a concentraciones bajas de estos contaminantes nocivos.

1. GENERAL INTRODUCTION

1.1. Pollution at sea

The oceans are vital for living organisms. They are home to millions of species, provide food and resources, regulate the climate and, basically, sustain life. However, during the last centuries, all water masses are receiving a lot of pressure from humans. Oceans are experiencing changes in physical, chemical and biological dynamics due to overfishing, aquaculture activities, chemical and physical pollution, human-induced rising temperatures, and coastal development (O'Hara and Halpern, 2022). Many marine species have disappeared as a consequence of overexploitation of marine resources and continuous habitat destruction. Nevertheless, degradation of the oceans is a global threat not only for marine environments but also for land-based ecosystems and species, including humans. Marine ecosystem services are diminishing due to ocean degradation and, therefore, human quality of life declines accordingly (Ruckhelshaus et al. 2013). These marine ecosystem benefits include food resources from fisheries and aquaculture, coastal protection in front of extreme weather events, erosion, and sea-level rise, among others (Ruckhelshaus et al. 2013).

Ocean pollution is one of the main reasons why the oceans are currently changing and, in fact, is considered a Global Planetary Boundary Threat (Rockström et al. 2009; Diamond et al. 2015; Villarrubia-Gómez et al. 2018). If not mitigated, pollution may exceed the limits of resilience of the oceans in the near future (Rockström et al. 2009). inadequate waste disposal and leaching of non-natural chemicals into the oceans are included in the hazards considered within this Planet Boundary Threat.

Pollution by waste includes discarded material from maritime traffic and transport, from fisheries and from mismanaged waste at land, where it enters into rivers, streams and wind currents to be finally dumped into the sea, particularly during storms and heavy rain events (Macías-Zamora, 2011; Pedrotti et al. 2022). Globally, Jambeck et al. (2015), calculated that 275 million tons of plastic waste were produced in 2010, from which 4.8-12.7 Mt were mismanaged and leaked into the ocean. A total of 367 Mt of plastics were produced globally in 2020, from which 55 Mt were produced only in Europe, accounting 15% of the global plastic production (Plastics Europe, 2021). Eriksen et al. (2014) estimated through modelling that more than 5.25 trillion plastic pieces floating at seas around the planet in 2014, most of which could be microplastics (MPs; Gago et al. 2016; Galloway et al. 2017). Far from experiencing a decrease, the continuation of current trends in production, consumption, and waste management of plastics would result in that up to 28 million tons of this material will be entering the oceans every year by 2025 (Jambeck et al. 2015). In fact, plastic littering is already predicted to be beyond the mitigation threshold (Borrelle et al. 2020).

Finally, hazardous chemicals, including plasticizers, pesticides, and heavy metals, reach the ocean either by spills, direct discharge into the sea, atmospheric deposition, and water runoff. They are also often transported along rivers that were polluted upstream (Cinnirella et al. 2013). However, knowledge about their distribution and concentrations in environmental matrices is still scarce. Regarding the variety of pollutants threatening the environmental health of the sea, it is important to precisely identify and quantify their distribution; aiming to plan effective mitigation and pollution prevention programmes.

1.2. Environmental status of the Mediterranean Sea

The Mediterranean Sea is an almost closed sea, only connecting to the Atlantic Ocean through the Strait of Gibraltar, and to the Red Sea by Suez Channel. A surface stream of cold water gets in the Mediterranean through the Gibraltar Strait and flows towards the East, where waters are warmer under normal conditions. Besides, the Eastern Mediterranean tends to accumulate greater concentrations of contaminants due to lack of water exchange with bigger basins. In spite of its limited size and water exchange, the Mediterranean Sea hosts from 4 to 18% of the known marine species in the world, of which more than one quarter are endemic, therefore showing the highest rate of marine endemism (Bianchi and Morri, 2000).

Climate and topography make the Mediterranean coasts ideal for human settlement. Approximately, 512 million people are permanent residents and 350 million tourists visit the area every year, mainly during the summer season (UNEP/MAP and Plan Bleu, 2020). Therefore, it has been heavily urbanized and is under great anthropogenic pressure. This process has considerably reduced the availability of permeable soil, which is essential for absorbing rainfall during the increasingly frequent flash-floods (Faccini et al. 2021). Also, sea-level rise and sand loss from beaches due to harbours nearby (Sanjaume and Pardo-Pascual, 2005), reduce buffer zones and coastal ecosystems' areas, as well as eliminate the protection and recreation function for humans (Molina et al. 2020; Faccini et al. 2021). In the sea, Mediterranean fish stocks have been depleted through overfishing; in fact, less than

20% of Mediterranean fisheries are exploited sustainably, in contrast to more than 60% in the Barents and Norwegian Sea (Froese et al. 2018).

The Mediterranean is known to accumulate a lot of plastic, including microplastics (Cózar et al. 2015; UNEP/MAP, 2015; Fossi et al. 2017; Sharma et al. 2021). The high human affluence to coastal-recreational areas seems to be the main reason for marine litter waste into the coastal and marine ecosystems (Constant et al. 2019). The seasonal nature of tourism and recreational use of these areas could explain the differential littering along the year, as suggested in previous studies (Bowman et al. 1998, Martinez-Ribes et al. 2007; Laglbauer et al. 2014; Munari et al. 2016; Pasternak et al. 2017; Prevenios et al. 2017; Vlachogianni et al., 2017). Land-based pollution sources, discarded fishing gear (Compa et al. 2019) and the continuous discharge of treated and untreated wastewater result in more than 64 million floating microplastic particles per km^2 in this sea (UNEP, 2020). Chemical additives in these contaminants also show up, eventually, in marine ecosystems. Specifically, phthalates, the most abundant plastic additives, have been found in the Mediterranean seawaters, sediments and biota (Guerranti et al. 2016; Paluselli et al. 2018a; Paluselli et al. 2018b; Savoca et al. 2018; Alkan et al. 2021; Schmidt et al. 2021, Ríos-Fuster et al. 2022). For instance, zooplankton sampled near a wastewater treatment plant (WWTP) from the Marseille Bay (north-western Mediterranean) presented high concentrations of phthalates, including the persistent DEHP (Schmidt et al. 2021).

The Mediterranean countries are also heavily exploited by intensive agriculture, which involves an intensive use of plant-protection products, including fertilizers and pesticides. Pesticides have been reported frequently along the Mediterranean coasts, including seawater, wetlands, deltas, and biota (Moreno-González et al. 2013). Pesticides enter into the marine system due to an inefficient or non-existent waste-water treatment, filtration through aquifers or leaking to running-water (Renau-Pruñonosa et al. 2020; Melendez-Pastor et al. 2021). Not only persistent pollutants are found, but also pesticides of rapid metabolism and degradation. Concentrations found can be explained by a continuous use and flow of pesticides from land to sea, which is common in the western Mediterranean (Gómez et al. 2007; Campo et al. 2013; Ccanccapa et al. 2016a; Ccanccapa et al. 2016b; Melendez-Pastor et al. 2021).

Heavy metals and trace elements in the Mediterranean can come either from natural or anthropogenic sources (Grousset et al. 1995) and through riverine input or atmospheric deposition (Guerzoni et al. 1999). The Mediterranean has been home to mining activities and sub-marine volcanoes that have contributed to the mercury load in the basin (Cinnirella et al. 2019). Heavy metals have been reported in sediments and biota (Storelli et al. 2005). Luckily, heavy metal pollution has been decreasing in the recent decades, thanks to new legislation and implementation of measures to reduce the waste of these pollutants (Reker et al. 2019).

In this scenario of multiple stressors, ocean conservation and sustainable management has become one of the top priorities in the United Nations Sustainable Development Goals (Sustainable Development Goals, United Nations 2030) and in the Convention on Biological Diversity (Alava, 2019). Efforts for marine protection have been made and legislative tools have been created in the European Union (EU) in the last decades. The Barcelona Convention for the Protection of the Mediterranean Sea and the EU Marine Strategy Framework Directive 2008/56/EC (MSFD) are the most potent legal tools currently for European Mediterranean countries. These regulations propose an ecosystem approach and aim to achieve or maintain a Good Environmental Status (GES) through legislation in the involved coastal countries through international European cooperation. Included in these tools, is the use of bioindicators species (Multisanti et al. 2022), such as marine turtles (Mattidi et al. 2017) and cetaceans (Fossi et al. 2018). The downside of this approach is that non-European Mediterranean countries are not included in the agreements and, therefore, efforts for marine protection are hindered.

1.3. Microplastics

One of the pollutants of concern for Mediterranean's environmental health, are microplastics. Microplastics are small plastic fragments that were first noticed and given this name by Thompson et al. (2004). Later on, the definition was refined and included an upper size limit of 5 mm to allow for better classification of these items (GESAMP, 2015). They can also be classified as primary or secondary, accounting for their origin. Primary microplastics include plastic materials that are manufactured that small on purpose, in order to be used as building blocks for bigger items, as erosive materials in industrial applications, or in personal care products (PCPs), among other uses. On the other hand, secondary microplastics are generated by the physicochemical weathering and degradation of bigger plastic items, which happens because of use or waste mismanagement (Cole et al. 2011; Hidalgo-Ruz et al., 2012; Andrady, 2017). Among the plastic materials that can shed microplastics, we can find consumer plastics but also paint, tires, fertilization and cleaning products, detergents, and sub-products from the petrochemical and gas industries (ECHA, 2022). In the European Economic Area, it is calculated that about 145 000 tons of microplastics are used every year (ECHA, 2022).

Since the introduction of the concept, it has become evident that microplastics are now ubiquitous in the environment (Wang et al. 2016; O'Hara and Halpern, 2022). They have been quantified in all kinds of marine ecosystems, from coastal areas to oceanic and deep waters, including marine trenches and remote areas (Jamieson et al. 2019). Therefore, they are also found in marine organisms along food webs, from primary producers to top predators (Wang et al. 2019). At a European level, they are being addressed by REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) regulations and by the MSFD.

Microplastics can be a threat to marine organisms. When ingested, they can block the gastrointestinal tract in small organisms and induce dietary dilution, eventually leading to malnutrition (Sëtala et al. 2014). More bothering is the fact that they can adsorb other chemicals from the water matrix and, therefore, potentially transfer them to marine organisms (Halle et al. 2020), thus potentially impacting negatively over all organisms' health (Avio et al. 2017).

1.4. Phthalate metabolites and pesticides

Since plastics are of common use and are normally present in every environment, the presence of plasticizer in seawater and in marine biota is expected. Plasticizers, such as phthalates, are added to polymers to give them convenient properties, such as flexibility, durability, and malleability (Rian et al. 2020; Hu et al. 2016; Hart et al. 2018). They are not covalently bonded to the main polymeric structure and, therefore, are easily leaked into the environment (Net et al. 2015; Hu et al. 2016). De Frond et al. (2018) estimated that about 190 tons of common 20 plastic additives entered the ocean in 2015, based on 7 plastic items of normal use by the population and their presence at sea (i.e., PET bottles and their caps, EPS, containers, cutlery, grocery bags, food wrappers and straws or stirrers).

Phthalates are quickly metabolized by organisms and their metabolites are excreted with urine (Asimakopoulos et al. 2016; Rian et al. 2020). However, Phthalates can induce antiandrogenic activity in humans and in model organisms (Pan et al. 2006; Hannas et al.

2011; Kumar et al. 2015; Sohn et al. 2016) by modifying hormone release (Hlisníková et al. 2020), but little is known about their effect on wildlife.

Pesticides are also common chemical species found in the marine environment, especially in areas where intensive agriculture is an important activity (Campo et al. 2013; Ccanccapa et al. 2016a; Ccanccapa et al. 2016b; Melendez-Pastor et al. 2021). Many of these pollutants are leaked into streams and rivers, reaching the sea through WWTPs, because these infrastructures are not prepared to remove them (Melendez-Pastor et al. 2021).

Pesticides can be both persistent and non-persistent. Persistent pesticides have a very low rate of metabolization and degradation, therefore, they can stay in environmental matrices for long periods of time (Pérez-Lucas et al. 2019). Their effects on organisms are well studied, unlike the effects of non-persistent pesticides, because these ones are considered safe or not present in non-target organisms. However, studies with human cohorts indicate that non-persistent pesticides are normally present in tissues and that they are suspicious of triggering thyroid dysfunctions (Campos and Freire, 2016). Yet, more research is needed in this direction, regarding that other studies point to non-effect exposure (Fernández et al. 2020; Yusà et al. 2022).

Phthalate esters and some pesticides are endocrine disruptors (EDs), which modify the hormonal response (Encarnaçao et al. 2019). They have antiandrogenic activity Hotchkiss et al. 2004; Pan et al. 2006; Hannas et al. 2011; Kumar et al. 2015; Sohn et al. 2016), promoting the disruption of hormone release (Hlisníková et al. 2020) and may bias population sex ratios. These effects could lead small populations to disappear if they were already threatened by other phenomena, such as genetic bottlenecks and/or climate change (Alava, 2020). EDs can also affect to sperm quality, decrease sperm count and, eventually, decreasing reproductive success (Di Lorenzo et al. 2020; Sharma et al. 2020). Studies of presence and effects of these two types of pollutants in wildlife, especially the non-persistent ones, are limited and also needed.

1.5 Species studied from the western Mediterranean

Loggerhead turtles (*Caretta caretta*, Linnaeus, 1758)

The loggerhead turtle (*Caretta caretta*, Linnaeus, 1758) belongs to the order Testudines and the family Cheloniidae. Globally, loggerhead turtles are classified as "Vulnerable" by the IUCN (International Union for the Conservation of Nature); although in the Mediterranean Sea, their status is of "Least concern" (Casale and Tucker, 2015). Despite this regional status, these species still face many different threats, including overfishing, bycatch and pollution. For this reason, the maintenance of this category by the IUCN is still dependent on conservation efforts (Casale and Tucker, 2015; Casale and Heppell, 2016, Rees et al., 2016).

As in other sea turtle species, hatchling loggerhead turtles rush into the sea right after they hatch. At early-juvenile stage of life they adopt an oceanic-pelagic distribution and move following dominant sea currents and winds (Abalo-Morla et al., 2018 and references therein). When they become juvenile and adults, they use to move towards coastal neritic habitats to feed (Bolten and Witherington, 2003); although recent studies have shown that part of them prefer to remain in pelagic areas even if the diversity of prey is poorer than in the coast (Ceriani et al. 2012; Eder et al. 2012; Mansfield et al., 2014, Eder et al., 2012). They are opportunistic foragers, feeding on a wide range of marine organisms. In the western Mediterranean, they prey on pelagic tunicates, crustaceans, molluscs, cephalopods and other invertebrates, but also on fish discarded by fisheries and longlain baits, as shown by stomach analyses and stablT isotopes (Tomás et al. 2001; Revelles et al. 2007; Cardona et al. 2012). They tend to occupy an intermediate position in the food web, above that of carnivorous cnidarians but lower than zooplanktivourous fish and crustaceans (Revelles et al. 2007).

Loggerhead turtles are good indicators of a good environmental status at a medium and basin scale (Bonano and Orlando-Bonaca, 2018; Fossi et al. 2018). They are long lived, which makes them prone to accumulate persistent pollutants over time (Finlayson et al. 2016), they tend to remain in the same foraging grounds for long periods of time and their diet is a good representation of organisms in the ecosystem of the area (Keller et al. 2013). More importantly, due to their opportunistic feeding nature, they constitute a good representation of the amount and characteristics of marine litter in the area they inhabit (Tomás et al. 2002; Doménech et al. 2019; Darmon et al. 2022). Besides, among the wild, protected species, loggerhead carcasses are relatively easy to access if there is a good relationship with local fishermen and authorities, as well as public awareness, which makes it possible to find specimens from bycatch and stranding events; as it is the case in the Valencian Community.

Striped dolphins (*Stenella coeruleoalba*, Meyen 1833)

The striped dolphin (*Stenella coeruleoalba*, Meyen 1833) belongs to the order Artiodactyla (aka Cetartiodactyla), and to the family Delphinidae. It lives in temperate and subtropical waters of all oceans (Aguilar and Gaspari, 2012) and it is the most abundant cetacean in western Mediterranean waters (Gómez de Segura et al., 2006; Aguilar and Gaspari, 2012). In this basin, it is found mainly as resident species in open waters beyond the continental shelf (Aguilar and Gaspari, 2012), although more neritic incursions and a potential habitat change have been recorded during the last two decades (Aznar et al., 2017; Fraija-Fernández et at. 2015). The IUCN classifies both the global and the Mediterranean subpopulation as Least Concern (Braulik, 2019; Lauriano, 2022). Mediterranean *S. coeruleoalba* seemed to feed mainly on oceanic cephalopod species and myctophid species (Blanco et al. 1995; Meissner et al. 2012; Aznar et al. 2017) but the diet may have shifted significantly in the last decades towards more neritic species such as juvenile hake (*Merluccius merluccius*) and southern shortfin squid (*Illex coindetii*) due to overfishing of their previous prey (Aznar et al., 2017).

Striped dolphins are predators high up in the marine food web. Like loggerhead turtles, they are widespread and abundant in the Mediterranean Sea and are characterized by a big range of motion. Because of these reasons, they are considered an indicator of healthy oceans (Schwacke et al., 2013; Fossi et al., 2018). However, their use as a bioindicator species is in doubt regarding that individuals are not easily accessible and, like marine turtles, their protection regime makes it difficult to work with live specimens.

Like other marine organisms, they can ingest microplastics directly from the water column, via trophic transfer, or during the inhalation at the air-water interface (Lusher et al., 2015; Fossi et al., 2018). Up to date, 60% of cetacean species are known to interact with plastics in seawater (Fossi et al. 2018) and microplastics have been documented in many species nowadays (table 7.1).

Jewel lanternfish (*Lampanyctus crocodilus*, Risso 1810)

Lanternfishes is a group of small pelagic fish that have bioluminescent photophores and inhabit all the oceans. They are found as part of the deep scattering layer and play a key role in carbon transfer from the sea surface layer to the bottom of the sea thanks to their diel vertical migrations (Fannelli et al. 2014). Lanternfish species belong to the family Myctophidae and are classified as "Least Concern" by the IUCN (Hulley, 2015). In the present thesis, we work with the jewel lanternfish, *Lampanyctus crocodilus* (Risso, 1810). *L. crocodilus* is found from 275 to 1000m depth (Froese and Pauly, 2000). They mainly feed on euphasiids and mysids, such as *Nemastocelis megalops* and *Meganyctiphanes norvegica*, but also on suprabenthic gammarids, pelagic decapods and gelatinous plankton, depending on the season and life stage (Fanelli et al. 2014). Therefore, they are considered secondary consumers. Their trophic level, based on food items, is 3.2 ± 0.39 se (Froese and Pauly, 2000). In turn, they are prey of the striped dolphins in the area and are very present in their diet in the last 10 years (Mateu et al. 2015; Aznar et al. 2017; Saavedra et al. 2022).

Lanternfish have been tested and proposed as small-scale bioindicators for the open-waters environment (Fossi et al. 2018). They are very abundant and one of the most ecologically relevant species of pelagic fish (Freer et al. 1019). Their microplastic content could be an indicator of microplastics' presence in bathypelagic waters. Sampling bathypelagic matrices is often complicated and expensive; however, myctophids are frequent by-catch species in trawling fisheries. Therefore, taking advantage of their capture and their small size and abundance, myctophids could be used to study different contaminants in the deeper layers of the ocean in a cheap and effective way.

Myctophids may increase the time that microplastics are bioavailable by ingesting them, either on purpose or by accident, when they are sinking towards the sea bottom and, therefore, these species may prevent microplastic sedimentation; introducing them in the food web. Once in the food web, microplastics can return to shallower layers of the sea thanks to myctophids' diel vertical migrations. Microplastics could be dangerous to this species due to their size and potential dietary dilution effect. That is, microplastics could block their gastrointestinal tract and trigger malnutrition and, eventually, failure to survive (Romeo et al., 2016; Fossi et al. 2018)

High amounts of microplastics have been detected in stomachs of mesopelagic fish species from the Atlantic Ocean (Davison and Asch, 2011; Lusher et al., 2016), and in some Mediterranean myctophids (*Electrona risso, Diaphus metopoclampus, Hygophum benoiti, Myctophum* *punctatum*; Romeo et al. 2016). The work presented in this thesis, in which 97 jewel lanternfish were analysed, adds up to the one carried out by Romeo et al. (2016) in the central Mediterranean basin during the period 2010-2014. With our results, we can compare different sub-basins increasing information for long–term monitoring studies.

2. PRESENT STUDY: AIMS AND OBJECTIVES

Aims

Within the current environmental framework, we aim to provide baselines about pollution levels, specifically on microplastics, plasticizers, pesticides and heavy metals, in wild understudied species in waters of the western Mediterranean Sea. For this purpose, we analysed different contaminants in several marine species that are abundant and have a key ecosystemic role in the area: loggerhead turtles (*Caretta caretta*, Linnaeus, 1758), striped dolphins (*Stenella coeruleoalba*, Meyen 1833), and jewel lanternfish (*Lampanyctus crocodilus*). Next, we describe the specific objectives.

Specific objectives

1. To find out whether loggerhead turtles in the Valencian Community are exposed to pesticides commonly used in the area. Furthermore, to show the variety of all pesticides that loggerhead turtles (*C. caretta*) can display in their tissues (fat and muscle) by performing a non-targeted chemical screening.

2. To find out whether metal pollution is a threat for loggerhead turtles' health, analysing the concentrations of the non-essential and potentially toxic metals, mercury (Hg), lead (Pb) and cadmium (Cd), in fat and muscle tissues from loggerhead turtles (*C. caretta*). Compare metal concentration between the Eastern and Western Mediterranean basins to elucidate possible spatial differences due to differential water exchange with bigger ocean basins. To study potential relationships between turtle size, developmental stage, and metal content.

3. To assess the exposure of loggerhead turtles (*C. caretta*) to phthalates, as indicator of the presence of these compounds at sea, by quantifying the concentration of phthalate metabolites in their livers. To find out potential temporal and geographical trends in

metabolite concentrations and potential relationships between phthalate metabolites' concentration and loggerheads' turtle size and developmental stage.

4. To quantify microplastics in beaches with different human use in Valencia region and to explore if the amounts of microdebris found could pose a threat to loggerhead turtles' nesting activity, including successful embryo development. Furthermore, to assess whether the amount of microplastics changes over the seasons, the proximity to urban areas and/or the regime of protection of the location. All of this taking into account a climate change scenario, in which increased nesting activity in the western Mediterranaen could be expected.

5. To assess whether a common cetacean species in the area, the striped dolphin (*S. coeruleoalba*), is affected by microplastic pollution. If that is the case, to identify the polymers of the microplastics found and to study possible temporal trends and relationships with dolphin's size and developmental stage.

6. To assess microplastics presence in the pelagic environment through the study of the bathypelagic jewel lanternfish (*L. crocodilus*), which are, in turn, an important part of the diet of the striped dolphins in the Spanish Mediterranean waters. Finally, to analyse whether fish body condition could account for the amount of microplastics ingested by this species.

The results of this PhD thesis will provide an overview of how exposed different Mediterranean wild species are to different pollutants of current concern. It will also provide an overview of the presence of different pollutants, and how are they distributed, in coastal and open waters of the westernmost part of the Mediterranean Sea, providing indirect and opportunistic sampling methods to assess marine pollution. The data collected will provide evidence for marine pollution risk assessment and management, and will provide information to help decision-makers to decide where and how to focus efforts to meet European environmental standards.

3. GENERAL MATERIALS AND METHODS

3.1. Study area

All samples used in this thesis were collected in the Valencian Community (East Spain, (from 40°31' N – 0°31' E to 37° 51'N – 0° 45'W, figure 1). This region is formed by three administrative provinces: Castellón (587 064 inhabitants), València (2 589 312 inhabitants) and Alicante (1 881 762 inhabitants); spanning geographically from north to south of Spai

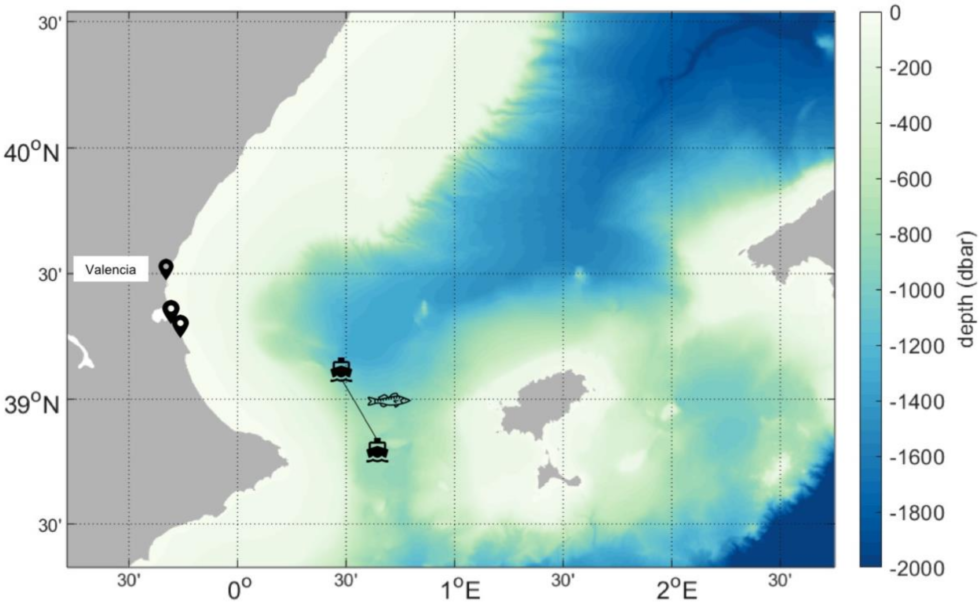


Figure 1. Study area. The map shows the bathymetry (blue scale), the beaches sampled for microplastics, the trawling ship's trajectory used for collecting myctophids (ships with the arrow). Fresh carcasses of loggerhead turtles and striped dolphins used in this thesis were found all over the coast, therefore they are not displayed in this map. The bathymetry base map was built on MatLab software by Daniel Fernández Román.

3.2. Common methods

In this thesis, a variety of methods were used, but some basic procedures were common throughout the different studies carried out. All biological samples were frozen at -20ºC as soon as possible during or after the sampling, and thawed at room temperature prior to the analyses. Due to the risk of external contamination in these procedures, contamination control measures were taken in every study.

All vacuum filtrations for microplastic separation were performed in type I laminar flow cabinets. These cabinets have a flow with positive pressure that stops external fibers from entering into the working environment. Microplastics shape, size and color classification in the different studies followed the guidelines proposed in the Marine Strategy Framework Directive (MSFD) of the European Union (EU) and the Harbour and Coasts Study Center (Spain, *Ministerio de Agricultura, Pesca, Alimentación y Medio Ambiente*, 2017).

All databases were built in Microsoft Excel and statistical analyses and visualizations were carried out in RStudio (RStudio Team, different versions from 2017).

Now, we present how the samples were obtained and a summary of the methods used specifically in each study.

3.3. Sample collection

Loggerhead turtles and striped dolphins' samples

Fresh biological samples from loggerhead turtles and striped dolphins were obtained through the Valencian Community stranding network for cetaceans and marine turtles, which is coordinated by public and private institutions, including the University of Valencia, l'Oceanogràfic and the *Conselleria de Agricultura, Desarrollo Rural, Emergencia Climática y Transición Ecológica* of the Local Government (*Generalitat Valenciana*). Samples were extracted during the necropsies of fresh carcasses, performed at the University of Valencia facilities.

In the case of loggerhead turtles (figure 2), 104 samples were used in total in this thesis. Of them, muscle and fat from 25 turtles collected between 2010 and 2016, were used to study metals and pesticides; and 79 liver samples collected between 2016 and 2021, were used to study the presence of phthalate metabolites.

In the case of striped dolphins, 43 individuals collected between 1988 and 2017 were used for the extraction of microplastics in their gastrointestinal tracts.

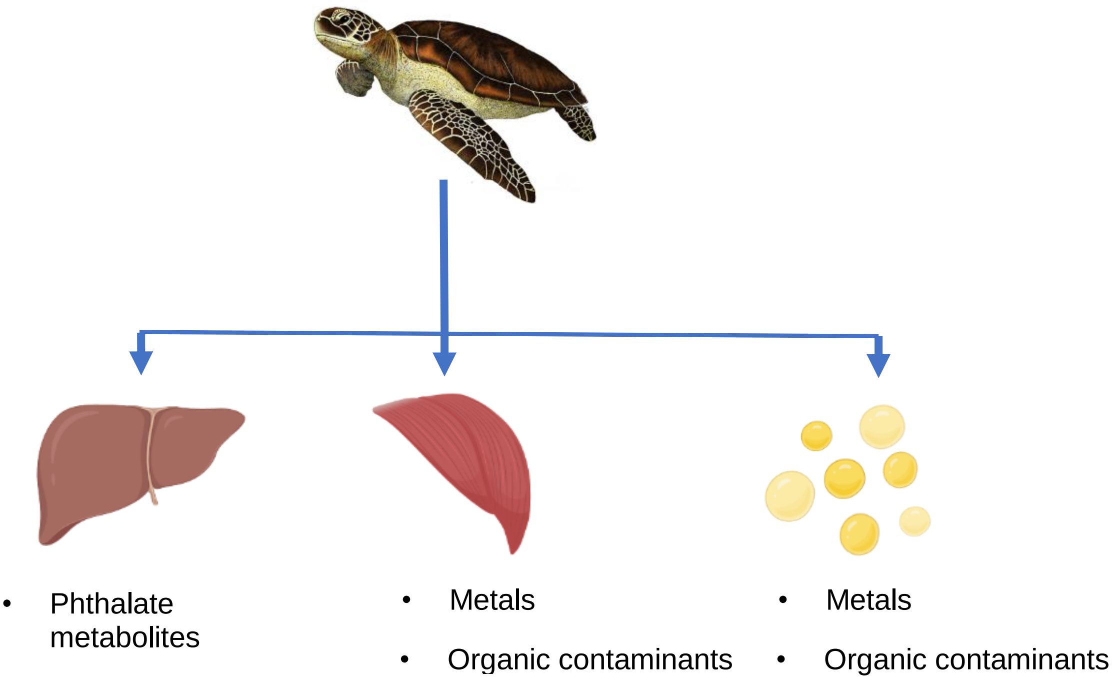


Figure 2. Summary of the samples collected during loggerhead turtles' necropsies for the different studies carried out in this thesis.

Microplastic analysis in sand

Sand samples were collected from three different beaches in Valencia province (Spain, figure 1): one of them is a urban beach located in Valencia city (El Cabanyal beach, 39°27'52.9"N 0°19'19.0"W), and the other two are located in El Saler municipality; l'Alcatí (39°19'13.1"N 0°17'53.5"W), of restricted access and La Punta (39°18'52.2"N 0°17'42.4"W), of forbidden access. Samples were collected in in two different seasons, summer and winter. At each sampling site, two depths were studied, the first centimeter of sand and sand at 40 cm below, typical depth of loggerhead turtle nests. Sampling at those depths allows testing for potential presence of microplastics nearby turtles' clutches and the possible effects in the incubation process, and also for microplastic sinking from the surface. Sand was stored in hermetic metallic containers until analyzed.

Jewel lanternfish samples

Specimens of jewel lanternfish (*Lampanyctus crocodilus*, N=94, Myctophidae family) were captured as by-catch by a trawling fishing vessel in the Ibiza Channel at approximately 300 m depth in June 2017 and collected by member of the Marine Zoology Unit (University of Valencia). These samples were obtained thanks to the collaboration between local fishermen and staff from the University of Valencia.

3.4. Laboratory procedures

Metals in loggerhead turtles

Muscle and fat samples from 25 loggerhead turtles were collected and stored frozen at -20ºC between 2010 and 2016.

Of each tissue, 0.3 g w. w. were digested overnight with 1 ml of nitric acid 68% and then diluted with 2 ml of distilled water. After homogenization, samples were centrifuged at 10000 rpm for 10 min and the clean portion of the extraction was analyzed for Cd, Hg and Pb by Inductively Coupled Mass Spectrometry (ICP-MS) with an Agilent equipment model 7900 following standard protocol used in the literature. Percentage of recovery was between 80% and 120%. Three replicates of each injection were performed to ensure quality of the analyses and the calibration curve has at least $r^2 > 0.995$.

Non-targeted analyses of organic chemicals in loggerhead turtles

Muscle and fat samples from 25 loggerhead turtles collected between 2010 and 2016 were studied using a non-targeted chemical analysis. Of each sample, 0.3 g w. w. were dissolved and homogenized by adding 1 ml of methanol sequentially until completing 3 ml. The remaining extract was then filtered with Teflon filters (ø = 0.45 µm, Chromacol 17-SF-45(T).45UM) and analyzed by Liquid Chromatography – Mass Spectrometry (LC-MS) with a UHPLC (Ultra High Performance Liquid Chromatography) system coupled to a hybrid quadrupole time-of-flight ABSCIEX TripleTOF™ 5600 LC/MS/MS System. We used an open access library that was originally used for urban wastewater analysis as a reference (Masiá et al., 2014), so we could also see whether contaminants in turtles reflect what is used in human industries and agriculture. Obtained data and chromatograms were assessed using PeakView™ software with the application XIC Manage.

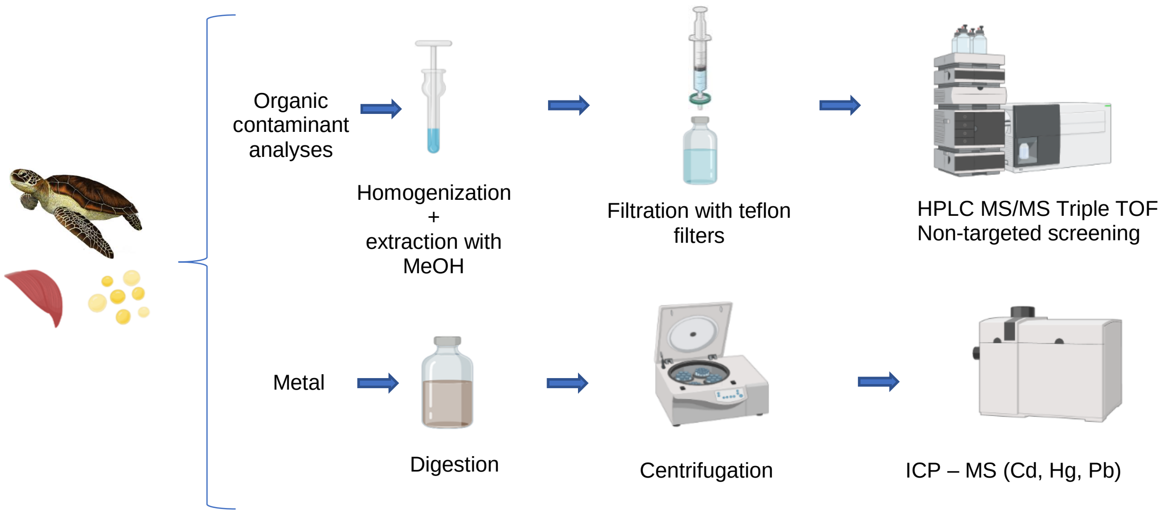


Figure 3. Summary of the methods used for metals and organic contaminant analyses with fat and muscle samples from loggerhead turtles (N = 25).

Phthalate metabolites in loggerhead turtles

Portions of 100 mg of 79 freeze-dried loggerhead turtles' livers were extracted with solid-phase-extraction (SPE). Samples were collected between 2016 and 2021. First, samples were fortified with 20 ng of internal standard (IS) phthalate metabolites' mix. To each sample, 600 µL of 1.0 M ammonium acetate were added, followed by 45 min ultrasonication. Thereafter, 600 µL of 1.0 M ammonium acetate with 22 units of β-glucuronidase were added and incubated during 12h at 37°C and 120 rpm. This deconjugation method allows to determine total concentrations of phthalate metabolites, that is, free and conjugated species of the target analytes (TA). After incubation, samples were centrifuged and their respective supernatants were diluted with 2 mL phosphate buffer. SPE was carried out with ABS Elut-NEXUS cartridges. The eluents were then concentrated to near dryness and reconstituted to 500 µL with acetonitrile: milli-Q water (1:9 v/v) for UHPLC-MS/MS analysis. Finally, UHPLC-MS/MS was carried out on an Acquity UPLC® I-Class system (Waters, Milford, CT, USA) coupled to a triple quadrupole mass analyzer (QqQ; Xevo TQ-S) with a ZSpray ESI ion source (Waters, Milford, CT, USA).

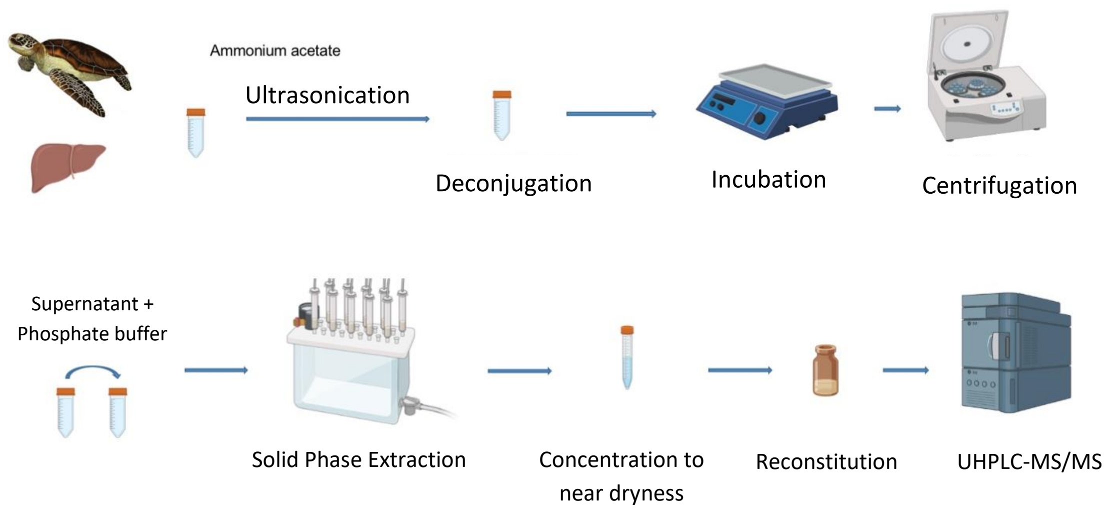


Figure 4. Summary of the phthalate metabolites' extraction and analysis method from loggerheads turtles' livers (N = 79) used in this study.

Microplastic analyses in sand from potential nesting beaches

Sand samples were dried at 50 °C for 48 h inside glass trays covered with perforated aluminum foil to allow water to evaporate. Microplastics were separated from the sediments by performing a density separation with a filtered supersaturated NaCl solution. Sand was mixed with the NaCl solution and left settling for a minimum of 6h of settling. After this time, samples were filtered under vacuum with Whatman borosilicate glass microfiber filters. The supernatant was then stirred with the sediment again and subjected to four consecutive filtrations in order to guarantee maximum microplastic recovery (Besley et al. 2017). Finally, all the filters were examined under a stereomicroscope (Leica MZ APO, 8–80x) and microplastics were quantified and classified accordingly.

Microplastic analyses in striped dolphins

The gastrointestinal tract (GIT) of 43 striped dolphins sampled between 1988 and 2017 were examined for microplastics following standard protocols used in literature. GITs' contents were washed through 200µm and 100µm nested sieves and introduced into glass bottles with a filtered 10% KOH solution for three weeks. After digestion, we filtered the solutions under vacuum using a Büchner filter and Whatman GF/C borosilicate glass microfiber filters. Then, the filters were dried for 24h at 60ºC and observed under a dissecting microscope (Leica MZ APO, 8–80×) to identify potential microplastics. Particles where the identification was more difficult were exposed to a hot needle to see whether they changed its shape, hence confirming its plastic composition (hot needle test, Hanke et al., 2013).

Figure 5. Summary of the methods for microplastics' extraction and identification from striped dolphins' digestive tracts. Striped dolphin original illustration: Jessica Harvey Carroll.

Spectrometric analyses of microplastics

Plastic polymers were identified by ATR FT-IR (Attenuated Total Reflection Fourier Transformed Infrared Spectroscopy) with an Agilent Technology Cary 630 spectrometer. Each sample was analyzed three times in order to ensure accuracy; and in each measurement, 8 scans were performed. We used ATR as the measurement mode and set the wavelength range was limited to 4000–650 cm^{-1}. The resulting spectra were identified thanks to the setup of a custom polymer library. Only matches above 70% with reference spectra were accepted as valid. Spectral analyses were carried out with Agilent Microlab.

Microplastic analyses in the jewel lanternfish

Myctophids were necropsied under a dissecting microscope (Leica MZ APO, 8 – 80x) and their gastrointestinal tracts were digested in KOH 10% 1:3 v/v for a week at room temperature. After digestion, samples were filtered under vacuum through a Büchner system equipped with Whatman GC/F borosilicate glass microfiber filters. These filters were then dried for 24h at 60ºC and, finally, they were carefully observed under the same dissecting microscope where dissections took place in search of microplastics.

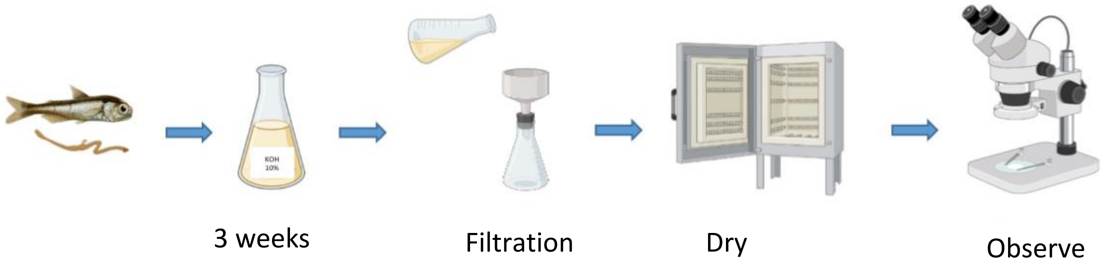


Figure 6. Summary of the methods for microplastics' extraction and identification from jewel lanternfish specimens (*L. crocodilus*) digestive tracts.

4. Exploring the presence of pollutants at sea: Monitoring heavy metals and pesticides in loggerhead turtles (*Caretta caretta*) from the western Mediterranean

Published in: Novillo, O., Pertusa, J. F., & Tomás, J. (2017). Exploring the presence of pollutants at sea: monitoring heavy metals and pesticides in loggerhead turtles (*Caretta caretta*) from the western Mediterranean. *Science of the Total Environment,* 598, 1130-1139. https://doi.org/10.1016/j.scitotenv.2017.04.090

Abstract

Marine turtles are considered good sentinel species for environmental assessment because of their long lifespan, feeding ecology, habitat use and migratory nature. In the present study, we assessed presence of cadmium, lead and mercury, together with organic pollutants, both in fat and muscle tissue of 25 stranded loggerhead turtles (*Caretta caretta*) stranded along the Valencian Community coast (East Spain) (43.7 ± 13.5 cm). Mean concentrations of Cd, Pb and Hg were 0.04 μg/g w.w., 0.09 μg/g w.w. and 0.03 μg/g w.w. in fat and 0.05 μg/g, 0.08 μg/g and 0.04 μg/g in muscle, respectively. These measures indicate a relatively low mean heavy metal concentration, which may be explained by juvenile size and developmental stage of the turtles analysed. A preliminary non-targeted analysis (using time-of-flight (TOF) technology), made for the first time in marine turtles, allowed to detect 39 different pesticides, most of them previously undetected in this species. Most of the organic substances detected are used in agricultural activities, and the use of 15 of them (38.5%) is not approved in the European Union. Our sample did not show any trend on pollutant contents in relation to turtle size or stranding location, probably because of the high diversity of pollutants found. However, the potential for a positive latitudinal gradient should be explored in future studies due to riverine inputs and high agricultural and industrial activities in the area. Despite the high diversity of pollutants found here, comparative studies of pollutants in other matrices at sea are needed to ascertain whether the loggerhead turtle is a good sentinel of chemical pollution in the western Mediterranean.

Introduction

Marine pollution is becoming a more serious problem day after day due to the massive amount of pollutants generated both by industrial and domestic activities. The Mediterranean Sea has limited exchange of water through the Strait of Gibraltar; hence, it is likely that contaminants appear at higher concentrations than in open oceans such as the Atlantic (Bucchia et al., 2015). Therefore, it is of vital importance to have good tools and strategies allowing pollution monitoring. Among these strategies, we can find biomonitoring, i.e., tracing compounds of concern by analysing contaminants in wildlife. Marine turtles are considered good sentinel species because of their long lifespan, feeding habits, their migratory nature, and their long periods of residency in foraging grounds (Keller, 2013 and references therein). Since they are a long-lived species, they are susceptible to accumulate high quantities of contaminants in their tissues (Caurant et al., 1999, Finlayson et al., 2016). Newborn and juvenile stages are more sensitive to the effect of environmental disruptors that may affect their development and health later in life. This is important in marine turtles having a long juvenile stage, when the probability of having deleterious effects increases (Brasfield et al., 2004).

The loggerhead turtle (*Caretta caretta*, Linnaeus, 1758) is a reptile belonging to the order Testudines and the family Cheloniidae. The species has been classified for many years as "Endangered" by the International Union for Conservation of Nature (IUCN). However, based on trends of nesting data, its status has been downgraded to "Vulnerable" globally, and to "Least concern" in the Mediterranean (Casale and Tucker, 2015). Despite the new status, these authors recommend keeping conservation efforts based on important threats, such as fisheries interaction and pollution. Both threats are reaching dangerous levels and work must be done to protect sea turtles in the Mediterranean area. Current by-catch levels in this basin are suspected to be unsustainable for preserving turtles' population (Casale and Heppell, 2016, Rees et al., 2016). The migratory behaviour of marine turtles, particularly of loggerheads, exposes them to contaminants from very different areas (Ragland et al., 2011). In the first stages of their life cycle loggerhead turtles are of oceanic-pelagic distribution. They typically move towards coastal neritic habitats during juvenile and adult stages although recent studies have shown that part of them prefer to remain in pelagic areas even if the diversity of prey is poorer in these waters (Hawkes et al., 2006, Mansfield et al., 2014, Eder et al., 2012). Hatchlings go through a pelagic state in which their movements are conditioned by sea currents and winds (Mansfield et al., 2014, Abalo-Morla et al., 2016). After this stage, loggerhead turtles from Mediterranean origin approach foraging neritic areas (Bolten and Witherington, 2003) such as the western Mediterranean, where they mix with individuals of Atlantic origin (Carreras et al., 2006, Carreras et al., 2011, Clusa et al., 2014, Clusa et al., 2016). After foraging, loggerhead turtles return to their nesting areas of origin (Bolten and Witherington, 2003 and references therein). Although dispersal and migration of loggerheads from eastern to western Mediterranean seems to be limited (Casale and Mariani, 2014 and references therein), the western Mediterranean hosts juveniles from both Mediterranean and Atlantic rookeries and seem to be segregated, with more turtles of Atlantic origin at the southern part of this basin (Clusa et al., 2014). This behaviour may hinder local conservation efforts, particularly in areas such as the Mediterranean, because even if some chemicals are banned in the European Union territory they could be intaken in other areas where environmental laws are different.

In general, reptiles may be more vulnerable to pollution than endotherm organisms, since their detoxifying systems and associated enzymatic activities are less developed (Schneider et al., 2015, Hopkins, 2005, Richardson et al., 2015). The loggerhead turtle is a generalist species, feeding in an opportunistic way. This characteristic makes them especially vulnerable to ingestion of residues. Ingestion and entanglement of marine litter, constituted mainly by plastic, is well documented in loggerhead turtles (Nelms et al., 2015, Rees et al., 2016), even in the Mediterranean (Tomás et al., 2001, Casale and Heppell, 2016). Plastic debris is of concern due to its persistence, toxic potential transporting potentially toxic chemicals, dispersion and ability of degrading key habitats (Nelms et al., 2015). Aerial surveys have shown high densities of loggerhead turtles in the western Mediterranean throughout the whole year (Gómez de Segura et al., 2006). In this area, gut content analyses revealed that juveniles of the species might consume large amounts of fishes that seem to come from discarded by-catch of western Mediterranean trawling fisheries (Tomás et al., 2001). They also consume pelagic tunicates, crustaceans, molluscs, cephalopods and other invertebrates mainly in benthic areas (Tomás et al., 2001, Revelles et al., 2007a). Besides, stable isotopes analyses have shown that they also consume considerable amounts of jellyfish such as the fried egg jellyfish (*Cotylorhiza tuberculata*) (Revelles et al., 2007b) and gelatinous plankton (Cardona et al., 2012 and references therein). Their trophic level is higher than that of carnivorous cnidarians but lower than that in zooplanktophagous fish and crustaceans (Revelles et al., 2007b).

Regarding all this information, the loggerhead turtle is an appropriate species to act as an environmental biomonitor of the seas (Keller et al., 2005, O'Connell et al., 2010, Ragland et al., 2011, Alava et al., 2011, Mattei et al., 2015). Firstly, their long lifespan makes them prone to accumulate contaminants throughout the years. Secondly, they uptake pollutants from the animals they consume, which also increases the probability of biomagnifying and storing hazardous substances for long periods. Finally, as they dive across the sea, they uptake substances from different territories, which could reflect zonal differences.

The location of the Valencian Community (East Spain, figure 1) is of special interest in relation to loggerhead sea turtle distribution and conservation. It is a region with a high degree of development and tourism along its coast. De la Nao Cape is likely the mainland boundary between Atlantic and Mediterranean loggerhead turtle stocks, with more Mediterranean turtles staying north to this cape and more Atlantic turtles remaining in the Algerian basin (Carreras et al., 2011, Clusa et al., 2014). North to this region outflows the Ebro River, the widest Spanish river flowing into the Mediterranean. This river is one of the most polluted in the country and therefore it is a potential source of pollutants flowing from land into this area (Campo et al., 2013, Ccanccapa et al., 2016b). In addition, main coastal currents flow north to south, bringing waters from the north-western Mediterranean and the area of influence of the Rôhne River in France (figure 4.1). Hence, it is of special interest to analyse geographical differences in sampled turtles, according to the stranding location.

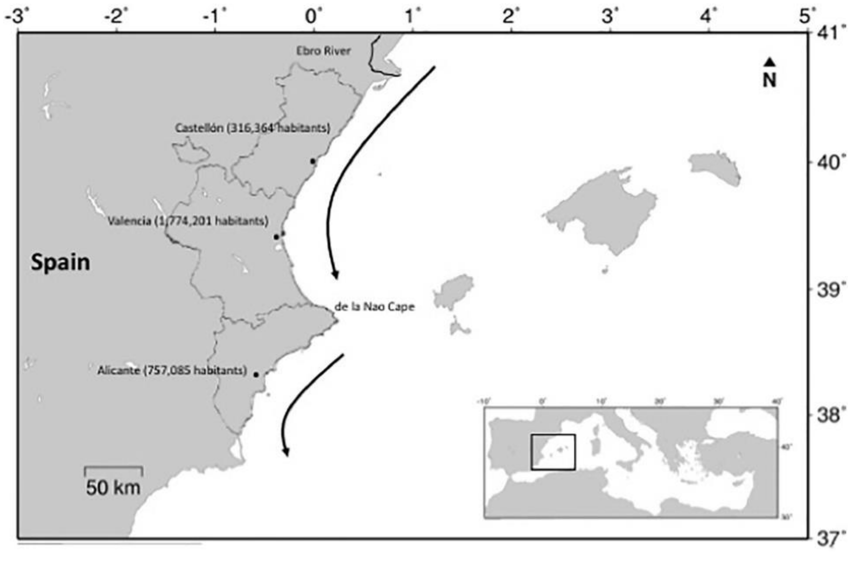


Figure 4.1. Study area within the Mediterranean Sea (inset), indicating the Valencia Community territory and its three provinces (Castellón, Valencia and Alicante, North to South) with the number of inhabitants of principal coastal metropolitan areas. Arrows indicate the main coastal sea currents,

according to Millot (1999). The indicated De la Nao Cape is the geographic division between North and South subareas considered here (see text for details). Map source: Seaturtle.org/maptool.

In the present study, we analysed cadmium (Cd), mercury (Hg) and lead (Pb) content in loggerhead turtles from the western Mediterranean. Their origin is mainly anthropogenic, and the three of them are systemic toxicants that are known to cause multiple damages (Tchounwou et al., 2014). Depending on their lipophilicity and other chemical characteristics, they can be accumulated differently in different tissues (Tchounwou et al., 2014). In addition, they accumulate trough time, thus their presence in organisms is an indication of environmental pollution. Contrary to many pesticides, metals are not easily degraded into less toxic compounds; hence, their effects are likely to be highly pernicious and long lasting, making them potentially toxic even at small concentrations (Storelli et al., 2005). Metals may have toxic and subtoxic effects on all developmental stages of the marine turtles' life cycle, from eggs to adults. A growing literature on heavy metal presence and toxicity in marine turtles exists (e.g., Kaska et al., 2004, Bucchia et al., 2015), although just one study analyses these pollutants in the species in western Mediterranean (García-Fernández et al., 2009). Concentration and toxicity of heavy metals varies depending on species' associated diet, stage of development, environmental conditions and anthropogenic sources of pollutants in the study area (Godley et al., 1999, Bucchia et al., 2015). We assessed heavy metals' concentration in loggerhead turtles' fat and muscle tissues, exploring whether those pollutants pose a risk to their health. We also explored ontogenetic variation, contaminants' mobility through ecosystems, and potential variation in metal concentration among loggerhead turtle Mediterranean stocks.

Marine turtles can be affected by organic pollutants as well. According to Finlayson et al. (2016), most of the studies have focused in searching the presence of POPs such as PCBs, PAHs and DDT metabolites' specifically (Mckenzie et al., 1999, Storelli et al., 2007, Lazar et al., 2011, Camacho et al., 2013a, Camacho et al., 2013b). However, many products proceeding from human activities can reach the sea, being toxic for marine species. To date only four studies have done chemical screening and a risk assessment in marine turtles, searching for specific pollutants (Lam et al., 2006, Van der Merwe et al., 2009, Keller et al., 2012, Dyc et al., 2015). Besides, there is just one non-targeted screening analysis that searches for a similar variety of agricultural products in marine animals' tissues and tests the methodology used (Munaretto et al., 2016). The present study consists of a preliminary assessment of organic contaminant content in marine turtles of the Mediterranean. A non-targeted screening analysis was performed to detect as many organic pollutants as possible by comparing results with a database previously used for analysing urban wastewaters in the area (Masiá et al., 2014).

Materials and methods

Sampling protocol

Samples from 25 loggerhead turtles were collected between 2010 and 2016 from turtles stranded or captured by fisheries along the coast and waters of the Valencian Community (East Spain, figure 4.1). Stranding management actions are coordinated by a stranding network which records dead or injured turtles and cetaceans beached ashore, floating dead or in a weakened condition, or bycaught and reported by fishermen. This network involves private and public institutions including rescue centres and aquaria in the region such as University of Valencia, l'Oceanogràfic and the Environment Office of the Local Government (Generalitat Valenciana). It is coordinated via a 24 h telephone hotline (Tomás et al., 2008). Necropsies of fresh carcasses are always performed following a detailed protocol at the facilities of the University of Valencia. During all the necropsies, Curved Carapace Length notch to tip (CCL) and other biometric variables were measured. CCL was used to establish relationships between contaminant concentration, size and developmental stage. Spearman correlations test was used to assess the link between metal content and stage of development (CCL).

Samples were taken from recently dead and stranded turtles. Subcutaneous fat and pectoral muscle tissue were collected from the specimens with sterile stainless steel material and stored in aluminium foil at − 20 °C until analysis. To assure reliability of the methods we prepared four blanks with distilled water, two for heavy metal analysis and two for organic pollutant analysis. For heavy metal analysis, 0.3 g wet weight of the samples were digested overnight with 1 ml of concentrated nitric acid 68% and then diluted with 2 ml of distilled water taking the protocol of Siscar et al. (2014) as a reference. After homogenization, samples were centrifuged at 10000 rpm for 10 min in order to get rid of impurities. Finally, metals in samples (Cd, Hg and Pb) were determined by Inductively Coupled Mass Spectrometry (ICP-MS) with an Agilent equipment model 7900, using rhodium and iridium as internal standards. To guarantee the quality of the analysis, possible fluctuations of the equipment and influence of the employed matrix were controlled. A fortified sample was prepared (5 ppb) and injected to test that percentage of recovery was in between 80 and 120%. Signal of RSD did not overcome 5%, indicating that the method was precise. Each injection was read three times and the calibration curve has at least $r2 > 0.995$. At the end of the sequence, an internal standard was analysed to guarantee that sensitivity of the equipment had not decreased.

For organic contaminant extraction, 0.3 g wet weight of the samples were dissolved and homogenized by adding 1 ml of methanol sequentially until reaching 3 ml in order to get rid of the organic part of the samples. As methanol was added the samples were crushed with glass instrumentation. The remaining liquid was then filtered with Teflon filters (ø = 0.45 μm, Chromacol 17-SF-45(T).45UM) and analysed by Liquid Chromatography – Mass Spectrometry (LC-MS) with a UHPLC system coupled to a hybrid quadrupole time-of-flight ABSCIEX TripleTOF™ 5600 LC/MS/MS System. Chromatography was performed on an Acquity UPLC BEH C18 Waters (1.7 μm particle size, 2.1 × 50 mm) column. The QqTOF was calibrated as recommended by the manufacturer in MS and MS/MS sensitivity mode. Electrospray ionization was operated in a positive mode, 5500 V of ion voltage, 80 V of declustering potential, 10 V of collision energy and 450 °C of temperature with curtain gas 30 (arbitrary units), 35 ion source gas 1 (GS1) and 35 ion source gas 2 (GS2). MS acquisition was performed using an information-dependent acquisition (IDA) that runs two experiments, one consisting of a full scan monitoring method over a mass spectrum range of 100–950 m/z; and an ion scan in which the system selects ions automatically. For this purpose, collision energy was fixed at 35 V and dynamic background subtract was activated. The system was calibrated using an external standard delivery system (CDS) which infuses calibration solution before sample injection. Besides, all the equipment in the laboratory is certified with the norm ISO 900, which guarantees a quality management system. In a targeted analysis, just a few known substances are detected by using reference standards, making the method costly and time consuming. By the contrary, with a non-targeted analysis like the one we performed with a TOF (Time of Flight) analyser it is possible to detect all the chemical species present in the samples. We used an open access library included also in the ABSCiex software. It was originally used for urban wastewater analysis as a reference (Masiá et al., 2014), so we could also see whether contaminants in turtles reflect what is used in human industries and agriculture. This library contains a total of 561 different pesticides. Results of LC-MS were expressed as intensity. Samples with intensities above a power of five were the only ones taken into account for the results, as they were considered to be present in the samples with the highest reliability. Obtained data and chromatograms were assessed using PeakView™ software with the application XIC Manage. XIC Manager identifies the compounds comparing results against a XIC Table containing 1212 pharmaceuticals, 561 pesticides, phenols and mycotoxins; and displays them in the chromatogram panel and in a table. These tables include name, formula, adduct/modification, retention time and width. Results also include found mass and mass error (ppm). A compound was accepted as present in the sample when three compulsory conditions were met. These conditions are: (1) error is below 5 ppm, (2) there is a good and evident isotope imprint and, (3) a chromatographic peak is present. Both ICP-MS and LC-MS were performed at "Servei Central de Suport a la Investigació Experimental (SCSIE)" of the University of Valencia. Legal status and chemical properties of the pesticides found were checked with the EU Pesticide Database (http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=homepage&language=EN; last visit: 4 July 2016) and Pesticide Properties Database of the British University of Hertfordshire (PPDB, Lewis et al., 2016), respectively.

Study area and geographical analyses

Mann-U Whitney and Kruskal-Wallis were used to test whether metal content was correlated with geographic and administrative areas. Geographic area was divided as "north" and "south" with De la Nao Cape chosen as a limit of separation according to regime of sea currents (figure 4.1) (Millot, 1999) and separation of turtle stocks. Administrative area was divided in the three formal provinces (Castellón, Valencia and Alicante, north to south) in which Valencian Community is divided (figure 4.1).

Results

Metal concentrations

All turtles analysed were of juvenile size (mean CCL ± SD = 43.7 ± 13.5 cm, range: 26.8–71.5 cm), except one of 71.5 cm of CCL that could be adult according to minimum nesting size in the Mediterranean (Casale et al., 2011 and references therein) and gonad examination during the necropsy. Metal concentrations (mean wet weight) of cadmium, lead and mercury were 0.04 μg/g, 0.09 μg/g and 0.03 μg/g in fat and 0.05 μg/g, 0.08 μg/g and 0.04 μg/g in muscle, respectively (table 4.1). Cadmium and mercury content was slightly higher in muscle than in fatty tissue and lead content was higher in fatty tissue than in muscle, although no significant differences were found (U Mann-Whitney, $p > 0.05$ in all cases).

Seven of the turtles showed high concentrations of lead: 1 both in fat and muscle (0.201, 0.119), 2 only in muscle (0.182, 0,117) and 4 only in fat (0.172, 0.237, 0.134, 0.115).

Table 4.1. Metal content (Cadmium [Cd], Lead [Pb] and Mercury [Hg], respectively) in loggerhead turtles' tissues analysed in the present study. Values in μg/g (wet weight).

Metals	Cd		Pb		Hg	
	Fat	Muscle	Fat	Muscle	Fat	Muscle
Mean ± SD	0.04 ± 0.02	0.05 ± 0.03	0.09 ± 0.05	0.08 ± 0.03	0.03 ± 0.02	0.04 ± 0.02
Range	0.02–0.10	0.01–0.17	0.04–0.24	0.04–0.80	0.00–0.08	0.01–0.10

No evidence of correlation between turtle size (CCL) and concentrations of any of the three metals was found, neither in fat nor in muscle samples (Spearman correlations, $p > 0.05$ in all cases) (figure 4.2). Mercury, cadmium and lead in fat and muscle were positively correlated ($r > 0.5$).

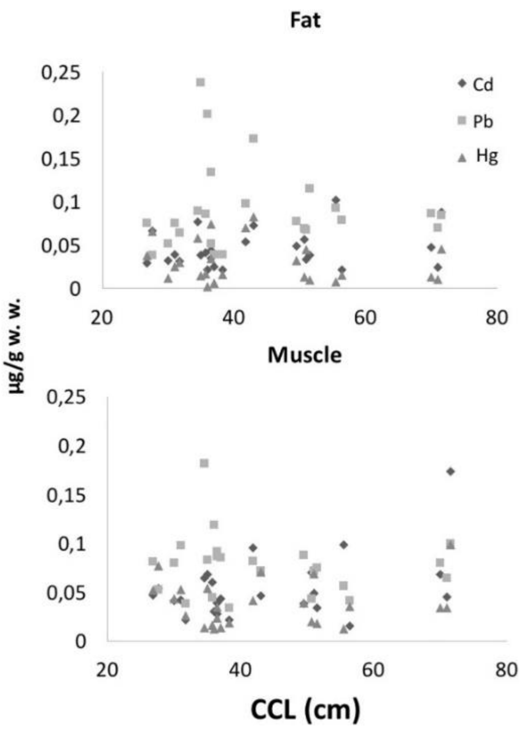


Figure. 4.2. Metal concentration in relation to size, expressed as CCL (cm), of loggerhead turtles stranded in the Valencian Community (East Spain).

Concerning spatial analysis, no significant geographical differences were found neither among provinces of the Valencian Community (Kruskal-Wallis, $p > 0.05$ in all cases) nor between the regions in which the study area was divided (North and South De la Nao Cape, U Mann-Whitney, $p > 0.005$ in all cases (figure 4.3).

Organic pollutants

All the analysed samples presented organic pollutants, that is, pesticides with an intensity above a power of five in the screening analysis. In total, 39 different pesticides were detected, of which 23 were found in fat tissue and 31 in muscle tissue of the turtles. Among these compounds, 17 are not approved for use in the EU and 3 are not assessed or have no data available (table 4.2). That is, 38.5% of the organic substances that seem to be incorporated in marine turtles' tissues are illegal. Among these 40 active compounds, 12 were

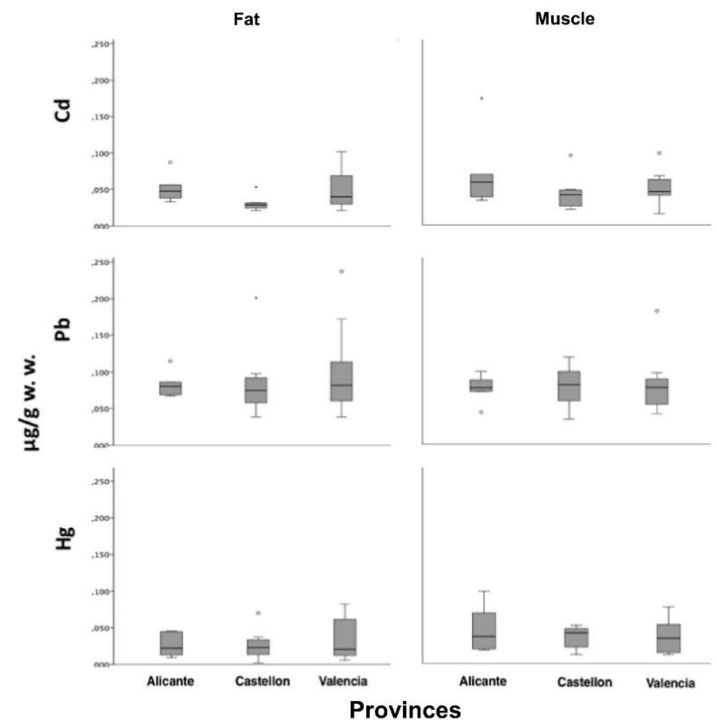


Figure 4.3. Median concentrations (µg/g w.w.) of three heavy metals (Cadmium [Cd], Lead [Pb] and Mercury [Hg]) recorded in Mediterranean loggerhead turtles according spatial distribution of strandings in the Valencian Community (N = 25). Administrative provinces South to North: Alicante (N = 8), Valencia (N = 10) and Castellon (N = 7). Boxplot: central line is median, upper lines of the boxes represent the 75th quartile, lower lines of the boxes represent the 25th quartile, end of whiskers are minimum and maximum in the 95th of cases, dots represent outliers.

insecticides, 11 were fungicides, 9 herbicides, 3 acaricides, 1 rodenticide, 1 antioxidant and 1 synergic substance.

Dodemorph and fenpropimorph were the most abundant pesticides reported in the analysis. Both are fungicides belonging to the morpholine class and were found in 56% of the samples (14 individuals). Their use is allowed in the EU. The mean intensity of dodemorph is remarkable (6,00E + 06), with some cases having intensities reaching a power of seven. Other important pesticides found were spiroxamine (28%), dodine (20%) and tralkoxydim (20% frequence of occurrence). Spiroxamine and dodine are also fungicides, belonging to the morpholine and guanidine class respectively, while tralkoxydim is a cyclohexanedione herbicide. Above 10% of the samples presented bitertanol (16%) and methomyl-oxime (12%), this last one being not a pesticide itself but a metabolite of the carbamate insecticide methomyl, approved by the EU. Use of bitertanol is not permitted in the EU (table 4.2). In one of the samples we found the highly toxic pollutant DDT (dichlorodiphenyltrichloroethane), an organochlorine contaminant with a high octanol-water partition coefficient (k_{ow} = 8.13 × 1006). Due to this property, it accumulates very easily and persists for a long time. Another highly lipophilic and bioaccumulable is the carbamate carbosulfan, which had a k_{ow} of 2.63 × 1007. Both compounds are illegal insecticides. The rest of the pesticides found did not have a high k_{ow}. Because of the nature of the screening analysis, exact concentrations could not be determined.

Discussion

Inferences from stranding data are subject to a number of caveats (Tomás et al., 2008), in part due to the advanced estate of decomposition of many carcasses. Nevertheless, in the present study only turtles in acceptable decomposition condition (either recently dead stranded turtles or bycaught dead turtles reported by fishermen) and in fair – excellent body condition were sampled.

Our study fills important gaps in the study of pollutants both at sea and on marine turtles. First, up to our knowledge, this is the first study in which a non-targeted screening of organic contaminants was carried out in marine turtles. Second, we consider for the first time the detection of mercury on marine turtles in the western Mediterranean. Third, there are few recent studies on pollutants in the Mediterranean (Andreani et al., 2008, García-Fernández et al., 2009, Lazar et al., 2011), and frequent monitoring is important considering the current levels of anthropogenic pollution. Our results have shown a wide diversity of pollutants, both heavy metals and pesticides, in loggerhead turtles from the western Mediterranean. Heavy metals at sea are mainly originated from anthropogenic activities in land, such as industry, smelting and vehicle traffic via rivers or atmospheric deposition (Nolting and Helder, 1991, Storelli et al., 2005, Acosta et al., 2010). For instance, leaded gasoline has been used for decades until it was banned at the beginning of the XXI century, and it has been reaching the sea principally by atmospheric deposition. Since this ban, lead has been decreasing in the environment over time (Storelli et al., 2005). Pesticides found in this preliminary screening analysis (table 4.2) come mainly from agricultural activities in land, although they can also come from products used in domestic gardening. Several of the organic compounds detected here have never been screened for any sea turtle tissue.

Heavy metals

All three metals studied here were detected in all the samples, indicating that these contaminants are widely dispersed in the study area. Lead was the most abundant metal, followed by cadmium and mercury. However, metal burden in the analysed turtles seems low compared with those of other studies elsewhere (table 4.3). It is important to consider that almost all the turtles sampled in this study were juveniles and that this could be the reason for the low metal content, since metals tend to accumulate with age and development (Jakimska et al., 2011). We did not find significant relationship between turtle size and heavy metal burden. Sample size did not include neither early stage of development (hatchlings and post-hatchlings) nor large adult individuals (> 75 cm CCL), hence a wider range of sizes should be analysed to explore relationships between body size contents and heavy metal concentrations.

Storelli et al. (2005) suggested that lead concentrations up to 0,5 μg/g (w.w.) in liver and kidney should not be considered toxic. In our study, none of the samples showed concentrations reaching this value because lead was measured in muscle and fat but not in the detoxifying organs, where their concentrations are much higher (D'Ilio et al., 2011). In fact, García-Fernández et al. (2009) obtained similar results to ours in muscle and besides they also analysed it in liver and kidney, finding concentrations above the limit suggested for

Table 4.2. Pesticides detected in fat and muscle samples from stranded loggerhead turtles in the Valencian Community. Lipophilicity, expressed as octanol-water partition coefficient (kow) and bioconcentration factor (BCF) are shown, as well as their legal status in the EU and intensity at which each compound was detected by the LC-MS screening. BCF < 100 = low potential, BCF 100–5000 = threshold for concern, BCF > 5000 = high potential; according to EPA standards.

Pesticide	**Use**	**Type**	**EU status**	**N**	**%**	**k_{ow} (20 °C, pH = 7)**	**RI muscle**	**RI fat**	**BCF (l/kg)**
Dodemorph	Fungicide	Morpholine	A	14	56	3.98×10^{04}	6,00E + 06	7,00E + 06	NA
Fenpropimorph	Fungicide	Morpholine	A	14	56	3.16×10^{04}	4,00E + 05	4,00E + 05	428
Spiroxamine	Fungicide	Morpholine	A	7	28	7.76×10^{02}	2,00E + 05	9,00E + 05	79
Dinoseb	Herbicide	Dinitrophenol	NA	5	20	1.95×10^{02}	5,00E + 05	6,00E + 05	192
Dodine	Fungicide	Guanidine	A	5	20	5.35×10^{-03}	9,00E + 05	7,00 + E05	16
Tralkoxydim	Herbicide	Cyclohexanedione	A	5	20	1.26×10^{02}	2,00E + 05	2,00E + 05	32
Bitertanol	Fungicide	Triazole	NA	4	16	$1,26 \times 10^{04}$	2,00E + 05	2,00E + 05	170
Methomyl-oxime	Insecticide	Carbamate	A	3	12	1.32×10^{01}	1,00E + 05	2,00E + 05	4,18
Acequinocyl	Acaricide	Unclassified	A	2	8	1.58×10^{06}	4,00E + 05	2,00E + 05	366
Buprofezin	Insecticide	Unclassified	A	2	8	8.51×10^{04}	–		509

Pesticide	Use	Type	EU status	N	%	k_{ow} (20 °C, pH = 7)	RI muscle	RI fat	BCF (l/kg)
Butoxycarboxim	Insecticide	Carbamate	NA	2	8	1.55×10^{-01}	–	2,50E + 07	LR
Butralin	Herbicide/growth control	Dinitroaniline	NA	2	8	8.51×10^{04}	–	1,00E + 05	1950
Carboxin	Fungicide	Oxathiin	A	2	8	2.00×10^{02}	–	8,00E + 04	13
Cycloxidym	Herbicide	Cyclohexanedione	A	2	8	2.29×10^{01}	6,00E + 05		LR
Famoxadone	Fungicide	Oxazole	A	2	8	4.47×10^{04}	1,00E + 06	–	3000
Furathiocarb	Insecticide	Carbamate	NA	2	8	3.98×10^{04}	1,00E + 05	1,00E + 05	92
Isoprocarb	Insecticide	Carbamate	NA	2	8	2.09×10^{02}	1,00E + 05	1,00E + 05	LR
Isoxaben	Herbicide	Benzamide	A	2	8	8.71×10^{03}	1,00E + 05	4,00E + 05	70,5
Metolcarb	Acaricide	Carbamate	NA	2	8	5.01×10^{01}	1,00E + 05	2,00E + 05	LR
Piperonylbutoxide	Unclassified	–	NC	2	8	7.76×10^{04}	2,00E + 05	4,00E + 05	NA
Propham	Herbicide	Carbamate	NA	2	8	3.98×10^{02}	–	5,00E + 04	LR
Benalaxyl	Fungicide	Acylamino acid	A	1	4	3.47×10^{03}	4,00E + 05		57

Pesticide	Use	Type	EU status	N	%	k_{ow} (20 °C, pH = 7)	RI muscle	RI fat	BCF (l/kg)
Bupirimate	Fungicide	Pyrimidinol	A	1	4	4.79×10^{03}	–	2,00E + 05	185
Carbosulfan	Insecticide	Carbamate	NA	1	4	2.63×10^{07}	2,00E + 05	–	990
Cyhalofop-butyl	Herbicide	Aryloxyphenoxypropionate	A	1	4	2.09×10^{03}	–	–	7,5
DDT	Insecticide	Organochlorine	NA	1	4	8.13×10^{06}	1,00E + 05	–	3173
Dimetilan	Insecticide	Carbamate	–	1	4	1.86×10^{00}	1,00E + 05	–	NA
Diphacinone	Rodenticide	Indandione anticoagulant	NA	1	4	7.08×10^{04}	2,00E + 05	–	NA
Diphenylamine	Antioxidant	Amine	A	1	4	6.61×10^{03}	1,00E + 05	–	NA
Etofenprox	Insecticide	Pyrethroid	A	1	4	7.94×10^{06}	–	2,00E + 06	NA
Fenazaquin	Acaricide	Quinazoline	A	1	4	3.24×10^{05}	–	5,00E + 05	699
Fenpropathrin	Insecticide/Acaricide	Pyrethroid	NA	1	4	1.10×10^{06}		2,00E + 05	1100
Fluometuron	Herbicide	Phenylurea	A	1	4	1.91×10^{02}	1,00E + 05	–	40,4
Flurprimidol	Growth regulator	Pyrimidinyl carbinol	NA	1	4	2.19×10^{03}	2,00E + 05	–	35,1

Pesticide	Use	Type	EU status	N	%	k_{ow} (20 °C, pH = 7)	RI muscle	RI fat	BCF (l/kg)
Formetanate	Insecticida/Acaricida	Formamidine	A	1	4	9.97×10^{-01}	–	1,00E + 05	1
Methacriphos	Insecticide	Organophosphate	NA	1	4	3.39×10^{01}	1,00E + 06	–	LW
Naled	Insecticide	Organophosphate	NA	1	4	1.51×10^{02}	1,00E + 05	–	598
Prosulfocarb	Herbicide	Thiocarbamate	A	1	4	3.02×10^{04}	1,00E + 05	–	700
Tebufenozide	Insecticide	Diacylhydrazine	A	1	4	1.78×10^{04}	5,00E + 05	4,00E + 05	70

A: approved by the EU. **LR:** low risk. **N:** number of turtles in which a pesticide was found. **NA:** not approved by the EU. **NC:** not considered. Information is available but is not classified as approved/not approved by the EU**. I:** intensity. **%:** frequency of detection of pesticides (N/Ntotal). **–:** not found. Information about these substances are not available neither the EU pesticide database nor in the PPBD of Hertfordshire University.

toxicity. Hence, the possibility that sampled turtles in our study were suffering subtoxic effects cannot be disregarded. There is previous evidence that lead is toxic to reptile species. For instance, Burguer (1998) studied its effect on hatchlings of slider turtle (*Trachemys scripta*), that displayed behavioural, developmental and survival alterations due to lead.

Cadmium content in the turtles was relatively low. Despite the presence of one turtle with high concentration of cadmium in muscle, we found no significant differences in concentrations among tissues. This sample corresponds to the biggest turtle analysed and, therefore, this concentration could be consequence of accumulation with age. Cadmium is known to accumulate with age, despite not biomagnifying (Gray, 2002) and appears to be especially abundant in the kidneys (Sakai et al., 2000). García-Fernández et al. (2009) suggest that this metal enters through dietary intake with high reliability because it does not appear to be regulated internally. However, Storelli et al. (2005) are more cautious and assure that it is risky to say that elevated cadmium content reflects dietary intake in an opportunistic organism such as the loggerhead turtle. Instead, they discuss that a high concentration of this element in loggerhead turtles could be given by a slow metabolization or physiological differences in comparison to other studied species (Caurant et al., 1999, Storelli et al., 2005). Dermal and respiratory routes are unlikely and lack demonstration. Few studies have focused on cadmium toxicity to turtles (Finlayson et al., 2016), but in terrestrial lizards' exposure of' eggs to cadmium affects negatively to hatchlings' growth, foraging efficiency, mortality, thyroid function and later reproduction (Brasfield et al., 2004). Turtles sampled in our study could have been experiencing deleterious effects since the levels found may produce health and developmental problems, according to García-Fernández et al. (2009). In marine fauna, this metal appears at high concentrations in organisms that feed on cephalopods (Caurant et al., 1999, García-Fernández et al., 2009). Loggerhead turtle feed on cephalopods in the western Mediterranean (Tomás et al., 2001, Revelles et al., 2007a); hence, this could be a potential way of accumulating cadmium in this species.

Mean mercury concentration was lower in fat tissue than in muscle tissue of the turtles, which agrees with the results found in other studies on the species (Storelli et al., 2005, Sakai et al., 2000). This may be explained by the higher affinity of methyl-mercury (MeHg) for sulfhydric (single bond SH) groups present in some proteins (Carvalho et al., 2002). In marine turtles, the organic form of mercury is the most abundant in muscle tissue, whereas the inorganic form is more abundant in the detoxifying tissues (D'Ilio et al., 2011).

Table 4.3. Comparison of our results (mean ± SD, µg/g w. w.) with previous studies in the area.

Study	n	Area	Cd muscle	Cd fat	Hg muscle	H fat	Pb muscle	Pb fat
Present study (2016)	25	W M	0.05 ± 0.03	0.04 ± 0.02	0.04 ± 0.02	0.03 ± 0.02	0.08 ± 0.03	0.09 ± 0.05
García-Fernández et al. (2009)	16	SW M	0.04 ± 0.03	NA	NA	NA	0.05 ± 0.05	NA
Storelli et al. (2005)	19	Adriatic and Ionian Seas	0.07 ± 0.03	0.08 ± 0.06	0.18 ± 0.02	0.04 ± 0.03	0.04 ± 0.03	0.09 ± 0.05
Maffucci et al. (2009)	29	S Italy	0.040 ± 0.04	NA	0.080 ± 0.06	NA	NA	NA
Franzellitti et al. (2004)	17	Adriatic Sea	0.36 ± 0.11	NA	NA	NA	NA	NA
Torrent et al. (2004)	78	N Atlantic Ocean (Canary Islands)	1.14 ± 0.28	NA	NA	NA	2.26 ± 0.5	NA
Kaska et al. (2004)	32	E M	0.71 ± 0.1.12	NA	NA	NA	0.48 ± 0.65	NA
Storelli et al. (1998)	12	Adriatic Sea	0.11 ± 0.13	NA	0.14 ± 0.11	NA	0.11 ± 0.03	NA
Caurant et al. (1999)	21	E Atlantic (Gironde Estuary, France)	0.08 ± 0.05	NA	NA	NA	NA	NA

Values expressed in dry weight were converted to wet weight assuming an 80% of water content for comparison purposes. (NA: not analysed; E: east, N: north, S: south, W: west, M: Mediterranean

For instance, MeHg in liver is decomposed and transformed in its less toxic form and eventually prepared for excretion (Storelli et al., 1998, Kampalath et al., 2006 in D'Ilio et al., 2011). High levels of Mercury may affect the endocrine and central nervous system subsequently disrupting reproduction, osmoregulation, prey location and interspecific communication, as found in several species of freshwater turtles (Bergeron et al., 2007). Unfortunately, studies about mercury toxicity in reptiles are very scarce and it is necessary to be careful when extrapolating results that could be misleading. Apart from that, allowed maximum level (ML) of mercury in commercial fishery products and fish muscle meat is 0.5 μg/g w.w., with the exception of some bigger species that are higher in the trophic chain, such as sturgeon (Acipenser species) and swordfish (Xiphias gladius), for which the limit is set to 1.0 μg/g w.w. according to the EU laws (EU Commission regulation (EC) No 1881/2006). Limits are based on species that are not in the top of the trophic chain (Wolfe et al., 1998) and, therefore, every organism that is over them could be accumulating higher amounts of pollutants and hence suffering toxic or sublethal effects. Nevertheless, turtles' metal content in the present study are generally below these levels.

Pesticides

The results of the screening of organic contaminants were surprising because of the diversity and nature of the pollutants found as well as their legal status. A comprehensive literature search revealed no previous studies on most of these pollutants in marine turtles. It is important to note that compounds found in the present study are also found in urban wastewater (Masiá et al., 2014). This is the first time that most of these compounds have been detected in animal tissue in the western Mediterranean.

Presence of pesticides was higher in muscle samples than in fat samples. This may occur because subcutaneous fat was scarce in some of the samples taken from the tissue bank. In spite of being from turtles in good body condition, these samples were not taken for toxicological purposes (see Material and methods) and therefore did not take fat specifically. Fat had to be removed carefully, inevitable taking some bits of skin away. Moreover, some pollutants are mobilized from fat before than in muscle, because fat is the first energy reserve to be used when there is a lack of energy. Hence, low persistent pollutants could have been underestimated in fat samples.

Fungicides were the most frequent organic pollutants found in the turtles analysed. According to pesticide databases consulted, dodemorph is allowed only in the Netherlands, Italy and Greece, but not in Spain. Fenpropimorph is allowed in every country of the EU except for Malta, Cyprus and Portugal. Despite the ban in certain countries, both fungicides appeared in 56% of the samples. Contrary to the general trend, both are moderately persistent, as they have a relatively high k_{ow} and therefore, they could have been assimilated far from the stranding site. In fact, Italy is in the migratory route of loggerhead turtles coming to the Spanish waters from the eastern Mediterranean (Casale and Mariani, 2014 and references therein). Spiroxamine was found in 28% of the samples and it is also allowed in almost every European country, except for Denmark, Finland, The Netherlands and Malta. As it is not persistent, its presence may reflect its use in territories close to the stranding site. Bitertanol is not approved in the EU because of its persistence and because it concentrates easily in organisms (see its bioconcentration factor, BCF).

Apart from fungicides, the herbicide dinoseb was found in 20% of the turtles in spite of not being permitted in the EU. In humans, its chronic exposure could lead to teratogenicity (neurodevelopmental problems and skeletal deformations). In addition, it is classified as a Possible Carcinogen by the EPA and it is an endocrine disruptor. It also affects the immunologic system, kidneys, liver and spleen, as well as being related with cataracts (EU Pesticides Database, PPDB). Therefore, the loggerhead turtle acts here as a bioindicator of the presence of this dangerous herbicide in western Mediterranean waters.

DDT (dichlorodiphenyltrichloroethane) was detected only in one of the turtles. However, the technique used is not sufficient to measuring this particular pesticide; hence, we cannot discard that its occurrence will be higher in western Mediterranean turtles. The affected turtle was a juvenile (CCL = 38.3 cm) female stranded in Penyíscola, in the north of the Valencian Community. As juvenile, this turtle might have been particularly vulnerable to this pesticide. Martínez-Gómez et al. (2012) showed that red mullets sampled at Delta del Ebro River (north to the study area) presented very high ΣDDT content. DDT could have been mobilized by the river from soil, where it had been used for years before it was banned. This could explain also its detection in the turtle. Otherwise, due to its persistence, the turtle could have acquired the DDT in waters of countries where it is still in use before reaching the study area. In loggerhead sea turtles, it has been shown to unbalance regulatory organs such as kidney and salt gland by altering the Na/K ratio (Camacho et al., 2013a).

In general, pesticides are not very lipophilic and hence they are not classified as highly pernicious for organisms, except for DDT. They tend to have a low k_{ow} and therefore their potential to bioaccumulate and persist is low (table 4.2). This chronic exposure could lead to sublethal effects that are of concern (McConnell and Sparling, 2010). However, we do not have data about effects of most of the pesticides on herpetofauna, and specifically on marine turtles. Pesticides could be also interacting between themselves and effects of mixtures remain unknown (Finlayson et al., 2016).

Pesticide ubiquity may indicate that there is a continuous flow of pesticides from land to sea, as it has been reported in the western Mediterranean (Campo et al., 2013, Ccanccapa et al., 2016a, Ccanccapa et al., 2016b), and thus loggerhead turtles are continuously exposed. As we said, we detected a wide diversity of organic pollutants in the turtles analysed here. Although they do not fully match with the pollutants reported in Campo et al. (2013) and Ccanccapa et al., 2016a, Ccanccapa et al., 2016b, most of them belong to the same category of pesticides. The fact that not permitted persistent pesticides appeared in the analysed loggerhead turtles means that, either the turtles came from countries that are not under European laws or that these pesticides may be used illegally. According to the European Commission (FCEC, 2015) there has been an increase in the trade of illegal and counterfeit Plant Protection Products (PPPs) in the last decade, reaching a 10% of the EU PPP market. However, if the pesticides found are of easy metabolization the most probable origin is non-approved use in Spanish Mediterranean provinces. Nevertheless, we do not have enough information about time of residence and migratory behaviour of the turtles, nor of bioaccumulation time in muscle and fat to determine exactly the origin of these pesticides.

Comparative biogeographical analyses

The high variability of pesticides found from individual to individual made impossible to determine a spatial distribution for organic substances body burden. Nevertheless, this fact could be indicative of a uniform distribution of these pollutants throughout the study area and surrounding regions.

We found no significant latitudinal pattern of metal contents in turtles stranded along the Valencian Community coast. Despite finding no significant differences, turtles from the south of the study area and Alicante province had higher cadmium content in general. This could be explained by a size effect more than a spatial effect, since turtles from the south were bigger than turtles found at the north of De la Nao cape (mean CCL ± SD = 57.4 ± 10.4 cm at south versus mean CCL ± SD = 39.1 ± 11.3 cm at the north). In fact, highest cadmium content was found in the largest individual, a turtle stranded in the Alicante province measuring CCL = 71.5 and containing 0.174 μg/g w.w. of this metal. However, the Rhône River (outflowing in south France) and the Ebro River are responsible for one third of the chemical pollution in the Mediterranean

Sea (Nolting and Helder, 1991, Gómez-Gutiérrez et al., 2006) and contaminant input from these rivers can be transported by main sea currents towards the south along the Spanish coast. Thus, further research controlling size effects and turtle movements within the basin, is needed to elucidate the potential effect of these rivers in pollutant burden on loggerhead turtles.

In our results, cadmium concentrations were similar to those found in turtles from southern Spain (García-Fernández et al., 2009) and Tyrrhenian Sea (Maffucci et al., 2005) (table 4.3), indicating that this pollutant may be present throughout this basin. Lead content in muscle resulted quite similar to those reported in the only study on pollutants in loggerhead turtles performed previously in the Spanish Mediterranean (García-Fernández et al., 2009). As we have mentioned before, our study is the first one analysing mercury presence in loggerhead turtles from the western Mediterranean, then it could be a reference for further comparative studies within this basin.

We also compared our results with studies carried out in eastern Atlantic Ocean, Adriatic Sea and eastern Mediterranean (table 4.3). Results for cadmium in muscle in our study are similar to those from Caurant et al. (1999) in the Gironde Estuary (west France). Surprisingly, results differed greatly from those obtained by Torrent et al. (2004) in the Canary Islands, with much higher values. Bucchia et al. (2015) compared contaminant burden in loggerhead turtles' blood from the waters surrounding the Canary Islands and from the Adriatic Sea, concluding that Mediterranean loggerhead turtles had higher concentrations of both organic contaminants (PCBs, organochlorines and PAHs) and metals (cadmium, copper, lead, mercury and zinc). Our results are slightly lower to those obtained by Storelli et al., 1998, Storelli et al., 2005 in the south Adriatic Sea. By the contrary, results in our study are very low when compared to studies performed by Franzellitti et al. (2004) in North Adriatic (Italy) and Kaska et al. (2004) in Turkey. In these last studies, values are of concern (table 4.3). Although full comparison is not possible because each study analysed some different metals in different tissues with different methodologies, we can observe a general pattern. It seems to be a positive eastward trend in pollutant burden in loggerhead turtles from the Atlantic, western and central Mediterranean to the north Adriatic Sea and eastern Mediterranean, indicating that in enclosed areas the turtles may accumulate more pollutants. This trend should be confirmed with further studies bearing in mind turtle size, since Kaska et al. (2004) included adult size turtles in their study.

Research needs

Loggerhead turtles may be more adequate than other marine turtle species as bioindicators of pollutants because of their position in the trophic chain. Higher levels of metals have been reported in this species than in the herbivorous green turtles (*Chelonia mydas*) (Godley et al., 1999). However, Storelli et al. (2005) pointed out that variability in their diet makes impossible to elucidate consistent trends. Never the less, thanks to the advances in knowledge about loggerhead turtle's feeding habits (Revelles et al., 2007a and b, Cardona et al., 2012) it is possible to test the reliability of this turtle as biomonitor. The species also makes possible to study contaminant transfer through the trophic chain and have a glance about contaminant content in non-commercial species, such as those eaten by the loggerhead turtle (Tomás et al., 2001).

The technique of non-targeted screening seems to be a good starting point in detecting the presence of several pollutants in loggerhead turtles. Regarding our results, there is a need of performing more screening analysis in different locations so as to detect and compare concentrations of as many as possible pollutants and chemicals found at sea and intaken by marine turtles. Nevertheless, organic matrices could cause interferences in detection methods. Further studies should include standards of specific pesticides in order to guaranty that detected compounds are indeed these compounds and also to obtain concentration in tissues.

The inexistence of toxicity thresholds for the loggerhead sea turtle makes it difficult to assess the potential risk of pollutants to this species. Hence, it is necessary to perform toxicity studies on marine turtles focusing on chronic exposure and sublethal effects of mixtures of pollutants with the help of in vitro techniques and biomarker analysis (Isaksson, 2010, Constantini et al., 2011, Rees et al., 2016, Beaulieu and Costantini, 2014). Such studies on environmental exposure and accumulation time performed on marine turtles would help in defining migratory pathways, as well as contaminant origin and mobility. Furthermore, there is a lack of comparative studies about pollutant body burdens in different foraging grounds (Finlayson et al., 2016). Comparisons have limitations because of the different endpoints assessed in different studies. Thus, it is important to standardize methods and establish endpoints that make these studies more reliable and useful. The information obtained can be used for doing accurate and informative environmental assessments, allowing detecting illegal substances, and studying the transfer of pollutants from land to sea.

Pollution constitutes a knowledge gap in marine turtles' conservation together with climate change (Hamann et al., 2010, Casale and Tucker, 2015, Rees et al., 2016). Without abandoning traditional conservation strategies, it is of vital importance to consider chemical pollution as a contributing factor for biodiversity decline.

References

Abalo-Morla, S., Tomás, J., Revuelta, O., Esteban, J. A., Eymar, J., Crespo, J. L., ... & Belda, E. J., 2016. Survival of reintroduced post-hatchlings loggerheads using satellite monitoring. *In 36th Annual Symposium on Sea Turtle Biology and Conservation*. Lima (Perú).

Acosta, J. A., Faz, A., & Martinez-Martinez, S., 2010. Identification of heavy metal sources by multivariable analysis in a typical Mediterranean city (SE Spain). *Environmental Monitoring and Assessment,* 169(1), 519-530. https://doi.org/10.1007/s10661-009-1194-0

Alava, J. J., Keller, J. M., Wyneken, J., Crowder, L., Scott, G., & Kucklick, J. R., 2011. Geographical variation of persistent organic pollutants in eggs of threatened loggerhead sea turtles (*Caretta caretta*) from southeastern United States. *Environmental Toxicology and Chemistry*, 30(7), 1677-1688. https://doi.org/10.1002/etc.553

Andreani, G., Santoro, M., Cottignoli, S., Fabbri, M., Carpenè, E., & Isani, G., 2008. Metal distribution and metallothionein in loggerhead (*Caretta caretta*) and green (*Chelonia mydas*) sea turtles. *Science of the total environment,* 390(1), 287-294. https://doi.org/10.1016/j.scitotenv.2007.09.014

Beaulieu, M., & Costantini, D., 2014. Biomarkers of oxidative status: Missing tools in conservation physiology. *Conservation Physiology*, 2(1), cou014-cou014. https://doi.org/10.1093/conphys/cou014

Bergeron, C. M., Husak, J. F., Unrine, J. M., Romanek, C. S., & Hopkins, W. A., 2007. Influence of feeding ecology on blood mercury concentrations in four species of turtles. *Environmental Toxicology and Chemistry*, 26(8), 1733-1741. https://doi.org/10.1897/06-594R.1

Bolten, A.B., Witherington, B. E. (Eds.), *Loggerhead Sea Turtles*, Washington, D.C., 2003. (352 pp.)

Brasfield, S. M., Bradham, K., Wells, J. B., Talent, L. G., Lanno, R. P., & Janz, D. M., 2004. Development of a terrestrial vertebrate model for assessing bioavailability of cadmium in the fence lizard (*Sceloporus undulatus*) and in ovo effects on hatchling size and thyroid function. *Chemosphere,* 54(11), 1643-1651. https://doi.org/10.1016/j.chemosphere.2003.09.030

Bucchia, M., Camacho, M., Santos, M. R., Boada, L. D., Roncada, P., Mateo, R., ... & Luzardo, O. P., 2015. Plasma levels of pollutants are much higher in loggerhead turtle populations from the Adriatic Sea than in those from open waters (Eastern Atlantic Ocean). *Science of the Total Environment*, 523, 161-169. https://doi.org/10.1016/j.scitotenv.2015.03.047

Burger, J., 1998. Effects of Lead on Behavior, Growth, and Survival of Hatchling Slider Turtles. *Journal of Toxicology and Environmental Health, Part A*, 55(7), 495-502. https://doi.org/10.1080/009841098158296

Camacho, M., Boada, L. D., Orós, J., López, P., Zumbado, M., Almeida-González, M., & Luzardo, O. P., 2013. Comparative Study of Organohalogen Contamination Between Two Populations of Eastern Atlantic Loggerhead Sea Turtles (Caretta caretta). *Bulletin of Environmental Contamination and Toxicology*, 91(6), 678-683. https://doi.org/10.1007/s00128-013-1123-3

Campo, J., Masiá, A., Blasco, C., & Picó, Y., 2013. Occurrence and removal efficiency of pesticides in sewage treatment plants of four Mediterranean River Basins. *Journal of hazardous materials*, 263, 146-157. https://doi.org/10.1016/j.jhazmat.2013.09.061

Ccanccapa, A., Masiá, A., Navarro-Ortega, A., Picó, Y., & Barceló, D., 2016. Pesticides in the Ebro River basin: occurrence and risk assessment. *Environmental Pollution*, 211, 414-424. https://doi.org/10.1016/j.envpol.2015.12.059

Ccanccapa, A., Masiá, A., Andreu, V., & Picó, Y., 2016. Spatio-temporal patterns of pesticide residues in the Turia and Júcar Rivers (Spain). *Science of the Total Environment*, 540, 200-210. https://doi.org/10.1016/j.scitotenv.2015.06.063

Cardona, L., Álvarez de Quevedo, I., Borrell, A., & Aguilar, A., 2012. Massive Consumption of Gelatinous Plankton by Mediterranean Apex Predators. *PLoS One*, 7(3), e31329. https://doi.org/10.1371/journal.pone.0031329

Carreras, C., Pascual, M., Cardona, L., Marco, A., Bellido, J. J., Castillo, J. J., Tomás, J., Raga, J. A., Sanfélix, M., Fernández, G., & Aguilar, A., 2011. Living Together but Remaining Apart: Atlantic and Mediterranean Loggerhead Sea Turtles (*Caretta caretta*) in Shared Feeding Grounds. *Journal of Heredity*, 102(6), 666-677. https://doi.org/10.1093/jhered/esr089

Carreras, C., Pont, S., Maffucci, F., Pascual, M., Barceló, A., Bentivegna, F., Cardona, L., Alegre, F., SanFélix, M., Fernández, G., & Aguilar, A., 2006. Genetic structuring of immature loggerhead sea turtles (*Caretta caretta*) in the Mediterranean Sea reflects water circulation patterns. *Marine Biology*, 149(5), 1269-1279. https://doi.org/10.1007/s00227-006-0282-8

Carvalho, M. L., Pereira, R. A., & Brito, J., 2002. Heavy metals in soft tissues of Tursiops truncatus and *Delphinus delphis* from west Atlantic Ocean by X-ray spectrometry. *Science of the total environment*, 292(3), 247-254. https://doi.org/10.1016/S0048-9697(01)01131-7

Casale, P., & Heppell, S., 2016. How much sea turtle bycatch is too much? A stationary age distribution model for simulating population abundance and potential biological removal in the Mediterranean. *Endangered Species Research*, 29(3), 239-254. https://doi.org/10.3354/esr00714

Casale, P., & Mariani, P., 2014. The first 'lost year' of Mediterranean Sea turtles: Dispersal patterns indicate subregional management units for conservation. *Marine Ecology Progress Series*, 498, 263-274. https://doi.org/10.3354/meps10640

Casale, P., Mazaris, A., & Freggi, D., 2011. Estimation of age at maturity of loggerhead sea turtles Caretta caretta in the Mediterranean using length-frequency data. *Endangered Species Research*, 13(2), 123-129. https://doi.org/10.3354/esr00319

Caurant, F., Bustamante, P., Bordes, M., & Miramand, P., 1999. Bioaccumulation of Cadmium, Copper and Zinc in some Tissues of Three Species of Marine Turtles Stranded Along the French Atlantic Coasts. *Marine Pollution Bulletin*, 38(12), 1085-1091. https://doi.org/10.1016/S0025-326X(99)00109-5

Clusa, M., Carreras, C., Pascual, M., Gaughran, S. J., Piovano, S., Avolio, D., Ollano, G., Fernández, G., Tomás, J., Raga, J. A., Aguilar, A., & Cardona, L. (2016). Potential bycatch impact on distinct sea turtle populations is dependent on fishing ground rather than gear type in the Mediterranean Sea. *Marine Biology*, 163(5), 122. https://doi.org/10.1007/s00227-016-2875-1

Clusa, M., Carreras, C., Pascual, M., Gaughran, S. J., Piovano, S., Giacoma, C., Fernández, G., Levy, Y., Tomás, J., Raga, J. A., Maffucci, F., Hochscheid, S., Aguilar, A., & Cardona, L., 2014. Fine-scale distribution of juvenile Atlantic and Mediterranean loggerhead turtles (*Caretta caretta*) in the Mediterranean Sea. Marine Biology, 161(3), 509-519. https://doi.org/10.1007/s00227-013-2353-y

Costantini, D., Monaghan, P., & Metcalfe, N. B., 2011. Biochemical integration of blood redox state in captive zebra finches (*Taeniopygia guttata*). *Journal of Experimental Biology*, 214(7), 1148-1152. https://doi.org/10.1242/jeb.053496

Dyc, C., Covaci, A., Debier, C., Leroy, C., Delcroix, E., Thomé, J. P., & Das, K., 2015. Pollutant exposure in green and hawksbill marine turtles from the Caribbean region. *Regional Studies in Marine Science*, 2, 158-170. https://doi.org/10.1016/j.rsma.2015.09.004

Eder, E., Ceballos, A., Martins, S., Pérez-García, H., Marín, I., Marco, A., & Cardona, L., 2012. Foraging dichotomy in loggerhead sea turtles *Caretta caretta* off northwestern Africa. *Marine Ecology Progress Series*, 470, 113-122. https://doi.org/10.3354/meps10018

EU Pesticide Database, n.d. *EU Pesticide Database* http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=homepage&language=EN (last visit: 4 July 2016).

Finlayson, K. A., Leusch, F. D., & van de Merwe, J. P., 2016. The current state and future directions of marine turtle toxicology research. *Environment international*, 94, 113-123. https://doi.org/10.1016/j.envint.2016.05.013

Food Chain Evaluation Consortium (FCEC)., 2015. Food Chain Evaluation Consortium (FCEC). Ad-hoc Study on the Trade of Illegal and Counterfeit Pesticides in the EU. *European Commission* (2015). Unpublished report. 8 pp.

Franzellitti, S., Locatelli, C., Gerosa, G., Vallini, C., & Fabbri, E., 2004. Heavy metals in tissues of loggerhead turtles (*Caretta caretta*) from the northwestern Adriatic Sea. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 138(2), 187-194. https://doi.org/10.1016/j.cca.2004.07.008

García-Fernández, A. J., Gómez-Ramírez, P., Martínez-López, E., Hernández-García, A., María-Mojica, P., Romero, D., ... & Bellido, J. J., 2009. Heavy metals in tissues from loggerhead turtles (*Caretta caretta*) from the southwestern Mediterranean (Spain). *Ecotoxicology and Environmental Safety*, 72(2), 557-563.https://doi.org/10.1016/j.ecoenv.2008.05.003

Godley, B. J., Thompson, D. R., & Furness, R. W., 1999. Do heavy metal concentrations pose a threat to marine turtles from the Mediterranean Sea? *Marine Pollution Bulletin*, 38(6), 497-502. https://doi.org/10.1016/S0025-326X(98)00184-2

Gómez-Gutiérrez, A. I., Jover, E., Bodineau, L., Albaigés, J., & Bayona, J. M., 2006. Organic contaminant loads into the Western Mediterranean Sea: estimate of Ebro River inputs. *Chemosphere*, 65(2), 224-236. https://doi.org/10.1016/j.chemosphere.2006.02.058

Gómez de Segura, A., Tomás, J., Pedraza, S. N., Crespo, E. A., & Raga, J. A., 2006. Abundance and distribution of the endangered loggerhead turtle in Spanish Mediterranean waters and the conservation implications. *Animal Conservation*, 9(2), 199-206. https://doi.org/10.1111/j.1469-1795.2005.00014.x

Gray, J. S., 2002. Biomagnification in marine systems: the perspective of an ecologist. *Marine Pollution Bulletin*, 45(1-12), 46-52. https://doi.org/10.1016/S0025-326X(01)00323-X

Hamann, M., Godfrey, M., Seminoff, J., Arthur, K., Barata, P., Bjorndal, K., Bolten, A., Broderick, A., Campbell, L., Carreras, C., Casale, P., Chaloupka, M., Chan, S., Coyne, M., Crowder, L., Diez, C., Dutton, P., Epperly, S., FitzSimmons, N., … Godley, B., 2010. Global research priorities for sea turtles: Informing management and conservation in the 21st century. *Endangered Species Research*, 11(3), 245-269. https://doi.org/10.3354/esr00279

Isaksson, C., 2010. Pollution and Its Impact on Wild Animals: A Meta-Analysis on Oxidative Stress. *EcoHealth*, 7(3), 342-350. https://doi.org/10.1007/s10393-010-0345-7

Kampalath, S.C. Gardner, L. Méndez-Rodríguez, J.A. Jay., 2006. Total and methylmercury in three species of sea turtles of Baja California Sur. *Marine Pollution Bulletin*, 52.,. 1784-1832.

Keller, J. M., Ngai, L., McNeill, J. B., Wood, L. D., Stewart, K. R., O'Connell, S. G., & Kucklick, J. R., 2012. Perfluoroalkyl contaminants in plasma of five sea turtle species: Comparisons in concentration and potential health risks. *Environmental Toxicology and Chemistry*, 31(6), 1223-1230. https://doi.org/10.1002/etc.1818

Keller, J. M., 2013. 11 exposure to and effects of persistent organic pollutants. *The Biology of Sea Turtles*, Volume III, 3, 285

Lam, J. C., Tanabe, S., Chan, S. K., Lam, M. H., Martin, M., & Lam, P. K., 2006. Levels of trace elements in green turtle eggs collected from Hong Kong: evidence of risks due to selenium and nickel. *Environmental Pollution*, 144(3), 790-801. https://doi.org/10.1016/j.envpol.2006.02.016

Lazar, B., Maslov, L., Romanić, S. H., Gračan, R., Krauthacker, B., Holcer, D., & Tvrtković, N., 2011. Accumulation of organochlorine contaminants in loggerhead sea turtles, *Caretta caretta*, from the eastern Adriatic Sea. *Chemosphere*, 82(1), 121-129. https://doi.org/10.1016/j.chemosphere.2010.09.015

Lewis, K. A., Tzilivakis, J., Warner, D. J., & Green, A., 2016. An international database for pesticide risk assessments and management. *Human and Ecological Risk Assessment: An International Journal*, 22(4), 1050-1064. https://doi.org/10.1080/10807039.2015.1133242

McConnell, L. L., & Sparling, D. W., 2010. 15 Emerging Contaminants and Their Potential Effects on Amphibians and Reptiles.

Mckenzie, C., Godley, B. J., Furness, R. W., & Wells, D. E., 1999. Concentrations and patterns of organochlorine contaminants in marine turtles from Mediterranean and Atlantic waters. *Marine Environmental Research*, 47(2), 117-135. https://doi.org/10.1016/S0141-1136(98)00109-3

Mansfield, K. L., Saba, V. S., Keinath, J. A., & Musick, J. A., 2009. Satellite tracking reveals a dichotomy in migration strategies among juvenile loggerhead turtles in the Northwest Atlantic. *Marine Biology*, 156(12), 2555-2570. https://doi.org/10.1007/s00227-009-1279-x

Mansfield, K. L., Wyneken, J., Porter, W. P., & Luo, J. (2014). First satellite tracks of neonate sea turtles redefine the 'lost years' oceanic niche. *Proceedings of the Royal Society B: Biological Sciences*, 281(1781), 20133039. https://doi.org/10.1098/rspb.2013.3039

Martínez-Gómez, C., Fernández, B., Benedicto, J., Valdés, J., Campillo, J. A., León, V. M., & Vethaak, A. D. (2012). Health status of red mullets from polluted areas of the Spanish Mediterranean coast, with special reference to Portmán (SE Spain). *Marine Environmental Research*, 77, 50-59. https://doi.org/10.1016/j.marenvres.2012.02.002

Masiá, A., Campo, J., Blasco, C., & Picó, Y., 2014. Ultra-high performance liquid chromatography–quadrupole time-of-flight mass spectrometry to identify contaminants in water: An insight on environmental forensics. *Journal of Chromatography A*, 1345, 86-97. https://doi.org/10.1016/j.chroma.2014.04.017

Mattei, D., Veschetti, E., D'Ilio, S., & Blasi, M. F., 2015. Mapping elements distribution in carapace of *Caretta caretta*: A strategy for biomonitoring contamination in sea turtles? *Marine Pollution Bulletin*, 98(1-2), 341-348. https://doi.org/10.1016/j.marpolbul.2015.06.001

Millot, C., 1999. Circulation in the western Mediterranean Sea. *Journal of Marine Systems*, 20(1-4), 423-442. https://doi.org/10.1016/S0924-7963(98)00078-5

Munaretto, J. S., May, M. M., Saibt, N., & Zanella, R., 2016. Liquid chromatography with high resolution mass spectrometry for identification of organic contaminants in fish fillet: screening and quantification assessment using two scan modes for data acquisition. *Journal of Chromatography A*, 1456, 205-216. https://doi.org/10.1016/j.chroma.2016.06.018

Nelms, S. E., Duncan, E. M., Broderick, A. C., Galloway, T. S., Godfrey, M. H., Hamann, M., Lindeque, P. K., & Godley, B. J., 2016. Plastic and marine turtles: A review and call for research. ICES Journal of Marine Science: *Journal Du Conseil*, 73(2), 165-181. https://doi.org/10.1093/icesjms/fsv165

Nolting, R. F., & Helder, W., 1991. Lead and zinc as indicators for atmospheric and riverine particle-transport to sediments in the Gulf of Lions. *Oceanologica Acta*, 14(4), 357-367.

O'Connell, S. G., Arendt, M., Segars, A., Kimmel, T., Braun-McNeill, J., Avens, L., Schroeder, B., Ngai, L., Kucklick, J. R., & Keller, J. M., 2010. Temporal and Spatial Trends of Perfluorinated Compounds in Juvenile Loggerhead Sea Turtles (*Caretta caretta*) along the East Coast of the United States. *Environmental Science & Technology,* 44(13), 5202-5209. https://doi.org/10.1021/es9036447

Pesticide Properties Database of the British University of Hertfordshire (PPDB), n.d. *Pesticide Properties Database of the British University of Hertfordshire (PPDB)* http://sitem.herts.ac.uk/aeru/ppdb/en/index.htm (last visit: 4 July 2016).

Camacho, M., Luzardo, O. P., Boada, L. D., Jurado, L. F. L., Medina, M., Zumbado, M., & Orós, J., 2013. Potential adverse health effects of persistent organic pollutants on sea turtles: evidences from a cross-sectional study on Cape Verde loggerhead sea turtles. *Science of the Total Environment*, 458, 283-289.

'Ragland, J. M., Arendt, M. D., Kucklick, J. R., & Keller, J. M., 2011. Persistent organic pollutants in blood plasma of satellite-tracked adult male loggerhead sea turtles (*Caretta caretta*). *Environmental Toxicology and Chemistry*, 30(7), 1549-1556. https://doi.org/10.1002/etc.540

Rees, A., Alfaro-Shigueto, J., Barata, P., Bjorndal, K., Bolten, A., Bourjea, J., Broderick, A., Campbell, L., Cardona, L., Carreras, C., Casale, P., Ceriani, S., Dutton, P., Eguchi, T., Formia, A., Fuentes, M., Fuller, W., Girondot, M., Godfrey, M., … Godley, B., 2016. Are we working towards global research priorities for management and conservation of sea turtles? *Endangered Species Research*, 31, 337-382. https://doi.org/10.3354/esr00801

Revelles, M., Cardona, L., Aguilar, A., Borrell, A., Fernández, G., & San Félix, M., 2007. Stable C and N isotope concentration in several tissues of the loggerhead sea turtle *Caretta caretta* from the western Mediterranean and dietary implications. *Scientia Marina*, 71(1), 87-93. https://doi.org/10.3989/scimar.2007.71n187

Revelles, M., Cardona, L., Aguilar, A., & Fernández, G., 2007. The diet of pelagic loggerhead sea turtles (*Caretta caretta*) off the Balearic archipelago (western Mediterranean): Relevance of long-line baits. Journal of the Marine Biological Association of the United Kingdom, 87(3), 805-813. *Scopus*. https://doi.org/10.1017/S0025315407054707

Richardson, K. L., Lopez Castro, M., Gardner, S. C., & Schlenk, D., 2010. Polychlorinated biphenyls and biotransformation enzymes in three species of sea turtles from the Baja California Peninsula of Mexico. *Archives of Environmental Contamination and Toxicology*, 58(1), 183-193.

Sakai, H., Saeki, K., Ichihashi, H., Suganuma, H., Tanabe, S., & Tatsukawa, R., 2000. Species-specific distribution of heavy metals in tissues and organs of loggerhead turtle (*Caretta caretta*) and green turtle (Chelonia mydas) from Japanese coastal waters. *Marine Pollution Bulletin*, 40(8), 701-709. https://doi.org/10.1016/S0025-326X(00)00008-4

Schneider, L., Eggins, S., Maher, W., Vogt, R. C., Krikowa, F., Kinsley, L., ... & Da Silveira, R., 2015. An evaluation of the use of reptile dermal scutes as a non-invasive method to monitor mercury concentrations in the environment. *Chemosphere*, 119, 163-170. https://doi.org/10.1016/j.chemosphere.2014.05.065

Siscar, R., Koenig, S., Torreblanca, A., & Solé, M., 2014. The role of metallothionein and selenium in metal detoxification in the liver of deep-sea fish from the NW Mediterranean Sea. *Science of the Total Environment*, 466, 898-905. https://doi.org/10.1016/j.scitotenv.2013.07.081

Storelli, M. M., Ceci, E., & Marcotrigiano, G. O., 1998. Distribution of heavy metal residues in some tissues of *Caretta caretta* (Linnaeus) specimen beached along the Adriatic Sea (Italy*). Bulletin of Environmental Contamination and Toxicology*, 60(4), 546-552.

Tchounwou, P. B., Yedjou, C. G., Patlolla, A. K., & Sutton, D. J., 2012. Heavy metal toxicity and the environment. *Molecular, clinical and environmental toxicology*, 133-164. https://doi.org/10.1007/978-3-7643-8340-4_6

Tomas, J., Aznar, F. J., & Raga, J. A., 2001. Feeding ecology of the loggerhead turtle *Caretta caretta* in the western Mediterranean. *Journal of Zoology*, 255(4), 525-532. https://doi.org/10.1017/S0952836901001613

Torrent, A., González-Díaz, O. M., Monagas, P., & Orós, J., 2004. Tissue distribution of metals in loggerhead turtles (*Caretta caretta*) stranded in the Canary Islands, Spain. *Marine Pollution Bulletin*, 49(9-10), 854-860. https://doi.org/10.1016/j.marpolbul.2004.08.022

van de Merwe, J. P., Hodge, M., Olszowy, H. A., Whittier, J. M., Ibrahim, K., & Lee, S. Y., 2009. Chemical contamination of green turtle (*Chelonia mydas*) eggs in peninsular Malaysia: implications for conservation and public health. *Environmental Health Perspectives*, 117(9), 1397-1401. https://doi.org/10.1289/ehp.0900813

Wolfe, M. F., Schwarzbach, S., & Sulaiman, R. A., 1998. Effects of mercury on wildlife: a comprehensive review. *Environmental Toxicology and Chemistry: An International Journal*, 17(2), 146-160. https://doi.org/10.1002/etc.5620170203

5. Phthalate metabolites in loggerhead marine turtles (*Caretta caretta*) from the Mediterranean Sea (East Spain region)

Novillo-Sanjuan, O., Sait, S.T., González, S.V., Raga, J.A., Tomás, J., Asimakopoulos, A.G. (In preparation). Phthalate metabolites in loggerhead marine turtles (*Caretta caretta*) from the Medirerranean Sea (East Spain region).

Presented in: Novillo-Sanjuan O., Sait S.T.L., Gonzalez S.V., Raga J.A., Tomás J., Asimakopoulos A.G. (2022). Presence of phthalate metabolites in livers of loggerhead turtles (*Caretta caretta*) from the Mediterranean Sea (East Spain). 7th Mediterranean Conference on Marine Turtles, Tetouan, Morocco. 18-24 oct. 2022.

Abstract

Phthalate esters are usually leached from plastics and personal care products that are often discharged or leaked into the sea. It is known that loggerhead turtles (*Caretta caretta*) from the western Mediterranean Sea are usually exposed to these plastics and other pollutants, potentially incorporating them into their organs. In order to assess phthalates occurrence in this protected species, we analysed phthalate metabolites in the livers of 79 turtles through solid phase extraction and UPLC-MS/MS analysis.

Phthalate metabolites were present in all the liver samples. Seven metabolites, including monomethyl phthalate (mMP), phthalic acid (PA), monoethyl phthalate (mEP), mono-n-butyl phthalate (mBP), mono-n-hexyl phthalate (mHxP), mono-n-nonyl phthalate (mNP), and mono-n-heptyl phthalate (mHepP), demonstrated detection rates (DRs) above 85%. The metabolites with the highest median concentrations were mDEP (38.9 ng/g d.w.), mEHP (22.2 ng/g d.w.), and mHxP (20.2 ng/g d.w.). The sum of the medians of phthalate metabolites concentrations that had DRs>85% showed a slight negative correlation between metabolites concentrations and size of the turtles (measured as curved carapace length), displaying an ontogenic dilution effect. This sum of the medians also showed a significant increase in phthalate metabolites concentrations from 2020 onwards ($p = 0.0005$), may be due to an increase in single-use plastic mismanagement following the Sars-Cov-2 pandemic or to uncontrolled wastewater discharges into the sea. Up to our knowledge, this is the first study analysing phthalate metabolites in marine turtle tissues.

Introduction

Phthalate esters (phthalates; PAEs) are widespread contaminants that are usually added to a variety of products including (but not limited to) herbicides, oil and gas drilling operations, and personal care products (Hart et al., 2018; Kassotis et al., 2016; Rocha et al., 2017; Tsochatzis et al., 2017). They provide plastics with elasticity, softness, and durability properties (Hu et al., 2016; Hart et al., 2018). They are not covalently bound to the polymeric structure of the plastics (Net et al., 2015; Hu et al., 2016), and consequently, they are prone to be leached into the environment. Phthalates have long half-lives (at neutral pH) that can reach up to the order of decades (Net et al., 2015). Nonetheless, their persistence in the environment is dependent on photo-oxidation and photolytic processes, sorption to other materials and biological uptake (Net et al., 2015). They have a relatively high volatilization potential and are easily transported by air and water currents (Net et al., 2015). Due to the continuous littering in coastal areas and the long-lasting nature of plastics in the marine environment, the ubiquitous occurrence of these additives in the oceans is expected.

Phthalates are well known endocrine disruptors, easily incorporated to biota, and consequently, impacting the reproduction and development of species (Oehlman et al. 2009; Hu et al. 2016). Endocrine disruption effects are demonstrated in both laboratory and epidemiological studies (Swan, 2008; Pan et al. 2006), although often using unrealistically high concentrations that are not actually found in the environment (Swan, 2008). Exposure to phthalates can induce antiandrogenic activity (Hotchkiss et al. 2004; Pan et al. 2006; Hannas et al. 2011; Kumar et al. 2015; Sohn et al. 2016), promoting the disruption of hormone release (Hlisníková et al. 2020), but actual effects on wildlife are understudied. Despite the global decline and vulnerability of reptiles, they are an understudied taxon in ecotoxicology (Gibbon et al. 2000; Weir et al. 2014). Experiments with fenced lizards (*Sceloporus occidentalis*) showed that phthalates tend to be retained in adipose and liver tissue (Weir et al. 2014). However, potential exposure effects in most of the reptile species currently are inferred from data exported from other animal groups, and therefore, conclusions cannot be reliably drawn.

In the Mediterranean, the loggerhead sea turtle (*Caretta caretta*) stocks are directly associated with the health status of the oceans, where declining populations often indicate habitat degradation (Bonano and Orlando-Bonaca, 2018; Fossi et al. 2018). Loggerheads have a key role in the marine food web and positively affect the diversity of species and dynamics of the benthic ecosystem (Bjorndal and Jackson, 2002). In the Mediterranean, they are categorized as Least Concern by the IUCN (International Union for the Conservation of Nature) but their population survival is still dependent on the conservation actions (Casale and Tucker, 2015). Loggerhead turtles ingest plastic litter in this sea basin due to their opportunistic feeding behaviour and the highly populated and touristic coastal areas that they tend to use for foraging (Doménech et al. 2019 and references therein). Also, turtle hatchlings swim towards high productivity convergence zones and oceanic fronts where litter tends to accumulate together with food (Nelms et al 2016; Darmon et al. 2017; Abalo-Morla et al. 2018; Cózar et al. 2021; Darmon et al. 2022). Their sex determination is dependent on the incubation temperature of the nests in sandy beaches (Standora & Spotila 1985, Mrosovsky 2002); which in a global warming scenario could lead towards feminization of future generations. These expected outcomes can be further worsened by endocrine disruption chemicals that could eventually lead to population collapse; there are reports of pseudohermafroditism in Mediterranean populations of loggerheads (Crespo et al. 2013), which highlight the endocrine disruption effects of chemicals such as phthalates.

To date, only few studies have addressed phthalates exposures in Mediterranean populations of loggerheads (Savoca et al. 2018; Savoca et al. 2021; Blasi et al. 2022). This can be attributed to the fact that phthalates biomonitoring is challenging due to high background contamination during the bioanalytical determination of those, often leading to overestimations and even false positive results (Net et al. 2015; Rian et al. 2020). Phthalates are rapidly metabolised by organisms, and therefore, the biomonitoring of their product metabolites, some of those only produced *in vivo* (Asimakopoulos et al. 2016; Rocha et al. 2017; Rian et al. 2020), can provide more reliable determination and improved exposure assessment (Hu et al. 2016). It is noteworthy that phthalate metabolites are also documented as standalone contaminants in environmental matrices themselves, as they are currently found widespread in river waters (Suzuki et al. 2001), marine and freshwater sediments (Otton et al. 2008; Blair et al. 2009), and seawaters (Blair et al. 2009).

With this background, the present study aimed to establish concentrations of phthalate metabolites in livers of a large sample of loggerhead turtles (n = 79) from the western Mediterranean Sea, collected along the coastal waters of Spain. The objectives were to: (1) investigate the occurrence of phthalate metabolites, and (2) assess the geographical patterns of the major phthalate metabolites in loggerhead turtles along the Valencian Community coast (East Spain); (3) establish associations among the target metabolites in the turtles; and (4) establish baseline concentrations needed for determining future trends in exposures. Sex differences and the relationship between liver concentrations and body size are also investigated. To our knowledge, this is the first study on the occurrence ofphthalate metabolites in loggerhead turtles.

Materials and methods

Chemicals and materials

All relevant information was reported by Rian et al. (2020). All target analyte standards (TAs; table S5.1) were purchased from Chiron AS (Trondheim, Norway). The isotopically labelled internal standards (table S5.1) were purchased from CDN Isotopes Inc. (Pointe-Claire, Canada). Individual stock solutions were prepared in methanol (MeOH) and stored at -20°C. Polypropylene (PP) tubes (15 mL) and amber glass LC vials (1.5 mL) were purchased from VWR International AS (Oslo, Norway). Ammonium acetate (≥ 98.0 % w/v), formic acid (98% v/v), acetic acid (≥ 99.0 % v/v) and β-glucuronidase from *Helix pomatia* (type HP-2, aqueous solution, ≥ 100,000 units/mL) were purchased from Sigma Aldrich (Steinheim, Germany). The Milli-Q water was prepared via a water purification system (Qoption, Elga Labwater, Veolia Water Systems LTD, U.K.). The remaining solvents were purchased from VWR Chemicals (Trondheim, Norway).

Sample collection and area of study

Loggerhead turtle samples (N = 79) were collected from dead specimens found stranded or bycaught by fisheries between the years 2016 and 2021 within the limits of the Valencian Community (East Spain; figure 5.1). The Valencian Community is formed by three administrative provinces: Castellón (587 064 inhabitants), València (2 589 312 inhabitants) and Alicante (1 881 762 inhabitants); spanning geographically from north to south of East Spain. The samples were collected by the Valencian Community Stranding Network. This network involves private and public organizations, including the University of València (València, Spain), l'Oceanogràfic aquarium (València, Spain) and the Environment Office of the Local Government (València, Spain). Most of the turtle liver samples came from turtles by-caught in artisanal fisheries, specifically by trammel nets and trawling fisheries, which are very common in the area (figure S1). The remaining samples came from freshly dead turtles reported stranded on beaches, bycaught by bottom-trawling fishing vessels, or found floating on sea surface (figure S1). Only one of the individuals was captured by longline fisheries (figure S1). Gas embolism was the cause of death for all by-caught individuals. The state of the carcasses was classified according to the Geraci and Lounsbury's (2005) criteria: 1. denoting "alive"; 2. "freshly dead"; 3. "starting decomposition, but the organs are basically intact"; 4. "advanced decomposition"; and 5. "mummified or skeletal remains only". Only the individuals belonging to states 2 and 3 were analysed to reflect actual contaminant concentrations, which are not affected by organ degradation. The Loggerhead turtles were mostly juveniles [mean Curved Carapace Length (CCL, notch to tip) ± (std. dev.) SD: 43.3 ± 12.9 cm; CCL range: 23.5 – 80.0 cm]. Only the biggest specimen (80.0 cm CCL) could be considered an adult, according to the size range established to estimate age, which considers 80 cm as the size at which sexual maturity is reached (Casale et al., 2011).

Sterile and stainless-steel materials were used in all the necropsies; and the CCL was measured. All samples were wrapped in aluminium foil and stored in the darkness at −20 °C. Sex was determined by direct observation of the gonads.

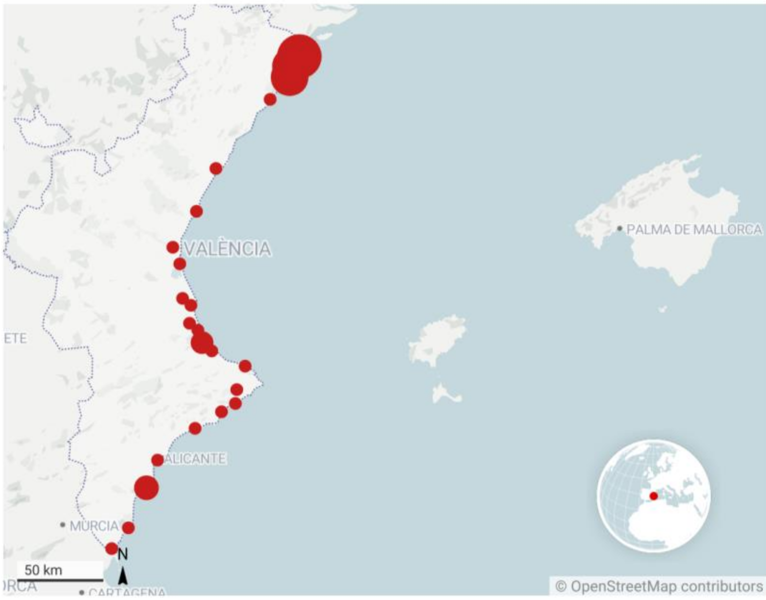


Figure 5.1. Locations of the Valencia Community where loggerhead turtles were found. The size of the dots is proportional to the number of turtles found in the marked location.

Sample preparation

Extraction of phthalate metabolites was performed according to Asimakopoulos *et al.* (2016) and Rian *et al.* (2020) with minor modifications. A portion of 100 mg (±25 mg) homogenized freeze-dried liver was added to a 15 mL PP tube, fortified with 20 ng internal standard mix (IS) and 600 µL 1.0 M ammonium acetate (aqueous solution) were added, followed by 45 min ultrasonication. Thereafter, 600 µL of 1.0 M ammonium acetate (aqueous solution) that contained 22 units of *β*-glucuronidase (prepared by spiking 25 µL of *β*-glucuronidase into 50 mL of 1.0 M ammonium acetate solution) were added, and the samples were digested for 12h in an incubator (37°C, stirred at 120 rpm) to determine total concentrations (free and conjugated species) of the TAs. Thereafter, the samples were centrifuged (10 min, 4000 rpm) and their respective supernatants were transferred to a new 15 mL PP tube and were diluted with 2 mL phosphate buffer (2 g sodium phosphate monobasic dehydrate dissolved in 100 mL milli-Q water with 1 mL orthophosphoric acid 85% v/v) prior to the loading SPE step. The ABS Elut-NEXUS cartridges were conditioned with 1.5 mL acetonitrile and equilibrated with 1.2 mL phosphate buffer followed by the sample loading step, and the cartridges were washed after the loading SPE step with 2 mL aqueous formic acid (1% v/v), followed by 1.2 mL milli-Q water. The cartridges were dried under vacuum for 15 min prior to elution with 1.2 mL acetonitrile followed by 1.2 mL ethyl acetate. The eluents were then concentrated to near dryness under a gentle stream of nitrogen. Finally, the eluents were reconstituted to 500 μL with acetonitrile:milli-Q water (1:9 v/v) for UPLC-MS/MS analysis.

UPLC-MS/MS analysis

The chromatographic separation was carried out on an Acquity UPLC I-Class system (Waters, Milford, CT, USA) coupled to a triple quadrupole mass analyser (QqQ; Xevo TQ-S) with a ZSpray ESI ion source (Waters, Milford, CT, USA). Separation was performed with an Acquity UPLC HSS T3 column (2.1 mm × 100 mm, 1.7 μm; Waters, CT, USA) serially connected to a SecurityGuard ULTRA C18 guard column (2.1 mm, sub-2 μm core-shell column; Phenomenex Inc., CA, USA) kept at 30°C. The mobile phase consisted of solvent (A) water containing 0.1% acetic acid (v/v) and (B) acetonitrile containing 0.1% acetic acid (v/v). The flow rate was 350 μL min^{-1} and the injection volume was 2 μL. The gradient elution began with 0% B, held for 0.5 min, and increased to 25% B in 0.5 min, held for 2.5 min (3.5 min), and increased to 35 % B in 0.5 min, held for 1.5 min (5.5 min), and increased to 50% B in 0.5 min, held for 1.5 min (7.5 min), and further increased to 90% B in 0.3 min, held for 1.7 min (9.5 min) before returning to initial conditions (0% B) and held for 1.5 min for a total run time of 11 min. Electrospray ionization was conducted in negative ionization mode (ESI-). Multiple reaction monitoring (MRM) mode was applied, and the optimal source settings of the tandem MS instrument were as follows: source temperature 150°C, capillary voltage 2.8 kV, desolvation temperature 350°C, and nebulizer gas pressure 6 bar.

Quality assurance and quality control (QA/QC)

The QA/QC procedures were similar to those reported by Rian et al. (2020). Procedural blanks were analyzed to monitor and control background contamination arising from laboratory materials and solvents. A 11-point standard solvent calibration curve ranging from 0.02 to 50.0

ng/mL (0.02, 0.05, 0.1, 0.2, 0.50, 1.00, 2.00, 5.00, 10.0, 20.0 and 50.0 ng/mL) was prepared and demonstrated a satisfactory regression coefficient for most TAs (R^2 > 0.998); mCPP and PA demonstrated lower regression coefficients at 0.96 and 0.94, respectively. Pre- and post- extraction spiked matrix samples were used as QA/QC samples and were prepared by spiking known concentrations of the TAs and ISs prior to and post sample preparation (extraction and clean-up). To monitor the drift in instrumental sensitivity and carry-over effects during analysis, a calibration check standard and a methanol solvent blank solution were injected, respectively, after the analysis of 25 consecutive samples. More details concerning method details and performance, are available in supplementary information (tables S5.1-S5.3).

Data and Statistical analysis

The distributions of the data (concentrations) were non-normal, and consequently, non-parametric tests were used for statistical treatment. Kruskal-Wallis test was used to search for significant differences between the concentration of the TAs and the independent variables of CCL, location, year and the carcass state. U-Mann-Whitney test was used to perform pairwise comparisons between the concentrations of the TAs and sex. Pearson correlations were used to explore associations. All data were recorded in Microsoft Excel (2021) and the analyses and data visualisations were carried out with RStudio (R Core Team, 2021) and ggplot2 (Whickham, 2016). Chromatographic data was acquired and processed with Intellistart, MassLynx and TargetLynx software packages (Waters, Milford, U.S.). Data analysis did not include censored data (i.e., non-detects; NDs). Concentrations were reported as ng/g dry weight (d.w.). Wet weight values were estimated dividing by a factor of 3.2, assuming a 68% of water content in the liver from Mediterranean loggerhead turtles (Mafucci et al. 2005).

Results and discussion

Liver concentrations of phthalate metabolites

Seven TAs, including monomethyl phthalate (mMP), phthalic acid (PA), monoethyl phthalate (mEP), mono-n-butyl phthalate (mBP), mono-n-hexyl phthalate (mHxP), mono-n-nonyl phthalate (mNP) and mono-n-heptyl phthalate (mHepP) demonstrated detection rates (DRs) ≥ 85% (table 1; N > 67). The metabolite with the highest median concentration was mono-n-decyl

Table 5.1. Occurrence of phthalate metabolites in liver samples of loggerhead turtles (N = 79). Concentrations are expressed as ng/g d.w., TA: Target Analytes.

TA	Detection rate (%)	Mean± SD	Median	Range
mMP	100	33.3 ± 163	12.0	1.56 - 1462
PA	98.7	74.6 ± 142	24.2	16.6 - 656
mEP	96.2	77. 6 ± 565	5.76	0.83 – 4939
mBP	89.8	5.51 ± 4.28	4.26	0.83 – 17.4
mHxP	87.3	27.6 ± 24.2	20.2	0.83 - 95.8
mNP	87.3	10.2 ± 6.84	9.72	0.83 - 33.1
mHepP	86.1	0.87 ± 0.18	0.83	0.83 - 2.08
mEHP	82.3	27.4 ± 20.0	22.2	4.19 - 90.5
mEHHP	82.3	1.19 ± 1.37	0.83	0.83 - 11.3
mCPP	79.7	15.5 ±17.2	9.92	3.33 - 87.7
mIBP	79.7	4.11 ± 11.3	2.21	0.83 - 91.4
mOP	75.9	5.29 ± 5.81	3.93	0.83 - 24.9
mBzP	74.7	14.5 ± 63.0	1.68	1.67 - 449
mEOHP	68.3	0.83 ± 0.01	0.83	0.83 - 0.89
mCHP	36.7	37.2 ± 184	0.83	0.83 - 995
mDeP	27.8	73.6 ± 98.4	38.9	0.55 - 417

phthalate [mDeP (38.9 ng/g d.w.)] followed by PA (24.2), mono(2-ehyl-1-hexyl) phthalate [mEHP (22.2)] and mHxP (20.2). The highest detection rates (DRs > 90 %) were found for mMP, mEP, and PA [analogues with shorter carbon chains or lower molecular weights (MW)]. Regarding their abundance and documented associations with the parent phthalates, lower MW metabolites are considered more reliable biomarkers (Hu et al. 2016). The presence of phthalate metabolites in the tissues is indicative of continuous exposure to phthalates, since the parent phthalates are rapidly metabolised; although the exact time frame of metabolization depends on factors such as developmental stage and route of exposure (Domínguez-Romero and Scheringer, 2019). The metabolites mono-n-pentyl phthalate (mPEP) and mono-iso-pentyl phthalate (mIPEP) were not detected.

The metabolite mEHP is formed by the hydrolysis of di(2-ethyl-1-hexyl) phthalate (DEHP, Fromme et al. 2007), which can be oxidized and form mono(2-ethyl-5-oxohexyl) phthalate (mEOHP) and mono(2-ethyl-5-hydroxyhexyl) phthalate (mEHHP) (Fromme et al. 2007; Asimakopoulos et al. 2016); mEHHP and mEOHP are secondary metabolites and are considered reliable markers of the presence of DEHP in animal tissues because they are produced *in vivo* (Asimakopoulos et al. 2016; Rian et al. 2020; Rocha et al. 2017). On the contrary, mEHP can be formed by abiotic processes, and therefore, it is not as reliable biomarker of exposure as mEHHP and mEOHP. The mEHP concentrations were higher by one order of magnitude (median: 27.4 ng/g d.w.) than those reported in bottlenose dolphin urine (median: 4.57 ng/mL, Dziobak et al. 2021) and in Mediterranean tuna muscle [1.73 ng/g (wet weight; w.w.) (Guerranti et al. 2016)], while approximately 3000 ng/g w.w. of the parent compound, DEHP, were reported in a *C. caretta* liver (Savoca et al.; 2018) and in *C. caretta* eggshells and yolk (medians of 12.5 ng/g and 7 ng/g w.w., respectively; Savoca et al. 2021). Herein, the concentrations of mEHP were higher than those of mEHHP and mEOHP (table 5.1) because the mEHP is the primary metabolite of DEHP and can also be formed by abiotic processes on the sample tissue itself.

PA was found in 98.7% of the analysed samples. PA is a generic hydrolysis end product of phthalates' metabolism; and therefore, it is a common biomarker of total phthalate exposure (Kluwe, 1982; Rian et al. 2020). Laboratory animal experiments documented that PA can induce oxidative stress (Bai et al. 2009; Song et al. 2014; Asimakopoulos et al. 2016). PA has been found also in liver of harbour porpoises (*Phocoena phocoena*) in Norway (Rian et al. 2020) at the same order of magnitude than in our turtles [(median concentrations in porpoises: 7.75 ng/g w.w; and in turtles: 24.2 ng/g d.w. (estimated 7.56 ng/g w. w.)].

mEP is a metabolite of diethyl phthalate (DEP), and it was found in 96.2% of the samples with a median concentration of 5.75 ng/g d.w. (estimated concentration: 1.80 ng/g w.w.) and it was on the same order of magnitude than the concentrations found in livers of harbour porpoises in Norway (Rian et al. 2020) and in the urine of bottlenose dolphins (*Tursiops truncates*) in Florida, USA; Hart et al. 2018; Dziobak et al. 2021). However, few samples with significant higher concentrations render this metabolite more variable in our study, in which concentrations ranged from 0.83 up to 4939 ng/g d.w. (estimated concentrations: 0.26 – 1543 ng/g w.w.) vs. the concentrations ranging from 2.62 to 17.4 ng/g w.w. reported in Rian et al. (2020). This difference can be attributed to the larger sample size herein, which implies a higher probability of finding extreme values in concentrations. On the other hand, other studies focused on quantifying the parent compounds. For instance, Vorkamp et al. (2004) analysed DEP in livers from polar bear (*Ursus maritimus*), minke whales (*Balaenoptera acutorostrata*), pilot whales (*Globicephala melas*) and ringed seals (*Phoca hispida*). Assuming that 100% of the parent DEP is metabolised to mEP by the organism, then the DEP concentrations (15.1 to 31.6 ng/g w.w.) in that study differs slightly from the mEP concentrations found herein. DEP was also found in Icelandic fin whale (*Balaenoptera physalus*) skin samples, but in much higher concentration (median = 303 ng/g d.w., García-Garín et al. 2022). DEP was also analysed in *C. caretta* tissues from Sicily, in which it was not detected (Savoca et al. 2018), and in *C. caretta* eggs from Linosa Island, in which DEP was detected in 17 samples (max.: 79 ng/g).

The isomers mBP (median: 4.26 ng/g d.w., estimated 1.33 ng/g w.w.) and mono-iso-butyl phthalate [mIBP (median: 2.21 ng/g d.w., estimated 0.7 ng/g w.w.)] were found in 89.8 and 79.7% of the samples, respectively. They are both products of the metabolic breakdown of di-n-butyl phthalate (DBP). The mBP and mIBP concentrations were found lower here than in harbour porpoises from Norway, where 25.2 and 30 ng/g w.w. were found, respectively (Rian et al. 2020). Savoca et al. (2018) determined a high concentration of DBP (2600 - 19000 ng/g w.w.) in liver and muscle of *C. caretta* from the Mediterranean Sea (Italy), while García-Garín et al. (2022) determined DBP median concentration of 303 ng/g d.w. in Atlantic fin whales. In another study, DBP was determined in *C. caretta*'s eggshells, yolk and albumen, with median concentrations of 26, 40.5 and 13 ng/g, respectively (Savoca et al. 2021). mMP, the major metabolite of dimethyl phthalate (DMP), was found in all liver samples (median: 12.0 ng/g d.w., estimated 3.76 ng/g w.w.) and in the same order of magnitude as in harbour porpoises' livers (0.34 – 8.72 ng/g w.w., Rian et al. 2020), and in liver and fat of other marine mammal species (2.5 – 8.4 ng/g w.w., Vorkamp et al. 2004). Neither mMP nor DMP were documented in marine turtle tissues before. mHxP, the metabolite of di-n-hexyl phthalate (DHxP), was found in 87.3% of the samples in this study and demonstrated a median concentration of 20.2 ng/g d.w. (estimated 6.33 ng/g w.w.), which was higher than that determined in the liver of harbour porpoises (detection rate of 45%, median of 0.63 ng/g w.w.; Rian et al., 2020). The parent compound, DHxP, was also reported in marine mammal livers (Vorkmap et al., 2004), and in human urine with detection rate of 59% (Frederiksen et al., 2020).

Lastly, median concentrations of mono-n-octyl phthalate (mOP) in turtle livers (3.93 ng/g d.w., estimated 1.22 ng/g w.w.) were in the same order of magnitude with those determined in harbour porpoises' liver (0.63 ng/g w.w., Rian et al. 2020), but lower than those reported in European eel muscle (*Anguilla anguilla*, mean = 82 ng/g d.w., Fourgous et al. 2016). Higher concentrations of mOP in eels could be explained by their habitat. Eels live in tighter bodies of water, while porpoises and marine turtles live in the open sea, where contaminant concentrations can be more diluted. The mOP precursor, di-iso-octyl phthalate (DIOP), was quantified in marine mammals' skin from the Mediterranean, including *Balenoptera physalus*, *Grampus griseus*, *Stenella coeruleoalba* and *Tursiops truncatus* (Baini et al. 2017). Although its detection rate was lower (47.4% in contrast to 75.9% in this study), concentrations in marine mammals were found to be higher than in the present study (306.7 – 3030.8 ng/g d.w. in contrast to a median of 3.93 ng/g d.w. herein).

To sum up, the median concentrations of the respective metabolites (mBP, mIBP, mBzP, mEHP, mEOHP and mEHHP, range: 0.83 – 449 ng/g d.w.) reported here are lower than the concentrations of the parent phthalates (assuming that the parent phthalates convert 100% to their respective metabolites) found in *C. caretta* tissues from the coasts of Sicily (DBP: 1250 – 19000 ng/g; DEHP: 2000 – 5900 ng/g approximately, Savoca et al. 2018). Concerning other species in the Mediterranean Sea, phthalate concentrations found here are generally lower to those reported in cetacean blubber by Baini et al. (2017) (mBzP = 32.1 ng/g d.w.; mBP: 780.1 – 983.7; mEHP: 463.7 – 1770; BBzP: 259.9 – 1629.1; DEHP: 1130 – 26068; and DIOIP: 306.7 – 3030.8 ng/g d.w); but higher than those concentrations found in tuna muscle samples (MEHP: 1.5 – 6.3 ng/g w.w.) analysed by Guerranti et al. (2016).

As it can be seen in the heatmap (figure 5.2), mDeP, PA, mHxP and mEHP, were generally found in higher concentrations than the remaining metabolites. It is noteworthy that the turtles that demonstrated the highest concentrations of PA (end product of phthalates metabolism), did not present high concentrations of the other metabolites, potentially due to their metabolic elimination to PA. Metabolic profiles vary depending on the species, and therefore, further studies concerning phthalates' metabolism in reptiles are deemed necessary.

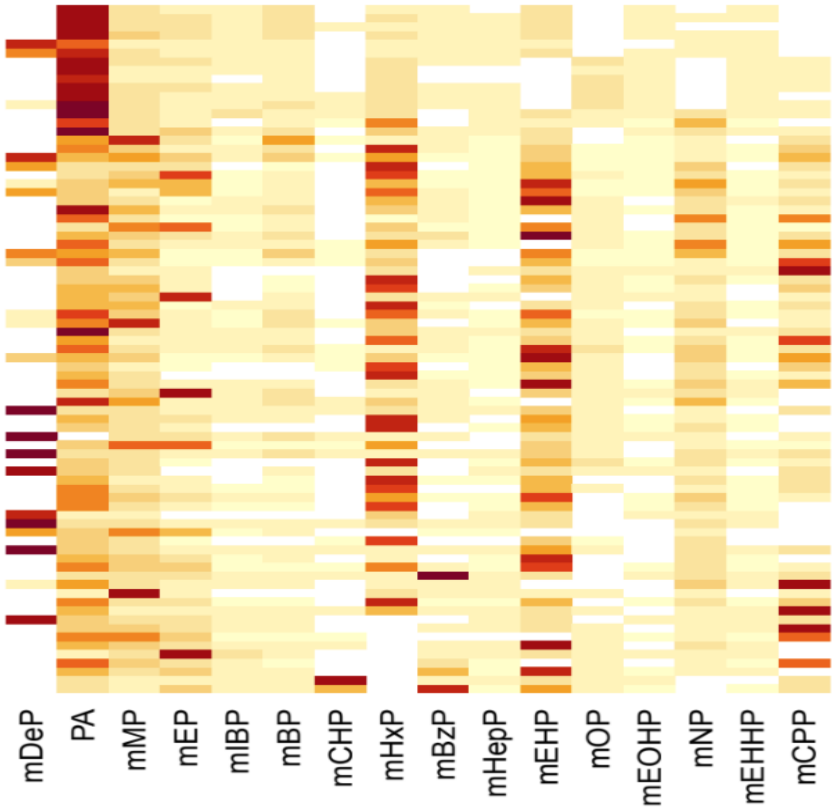


Figure 5.2. Heatmap of phthalate metabolites' concentrations in the livers of loggerhead turtles. Each row represents a sample and each column represents a phthalate metabolite.

Correlations (the probability value of $p < 0.05$ was set for statistical significance) between phthalate metabolites were presented in figure 5.3. The correlation between mBP and mHxP (r= 0.68) indicated a concomitant source of exposure. This can be attributed to the fact that the parent compounds of mBP and mHxP are co-occurring in many materials and consumers' goods. mEHHP was positively correlated with PA (r = 0.59), indicating a potential metabolic relationship between those. The sum of the medians of the phthalate metabolites with DRs > 85% (12 metabolites, Σ_{12}PhMet); including, in descent order, mMP, PA, mEP, mBP, mHxP, mNP, mHepP, mEHP, mEHHP, mCPP, mIBP, and mOP) demonstrated stronger correlations with PA (r = 0.58), mHxP (r = 0.51), mNP (r = 0.46) and mEHP (r = 0.46).

The sum of the medians of mEHHP and mEOHP (ΣmeHHP+meOHP), both secondary metabolites of DEHP, were calculated as well, and demonstrated positive correlations with mMP (r = 0.59), mEP (r = 0.89), and mIBP (0.91), which may indicate concomitant sources of exposure for DEHP, DMP, DEP and DIBP, respectively. Weaker correlations were found with mEHP (r= 0.3) and mDeP (r= - 0.12).

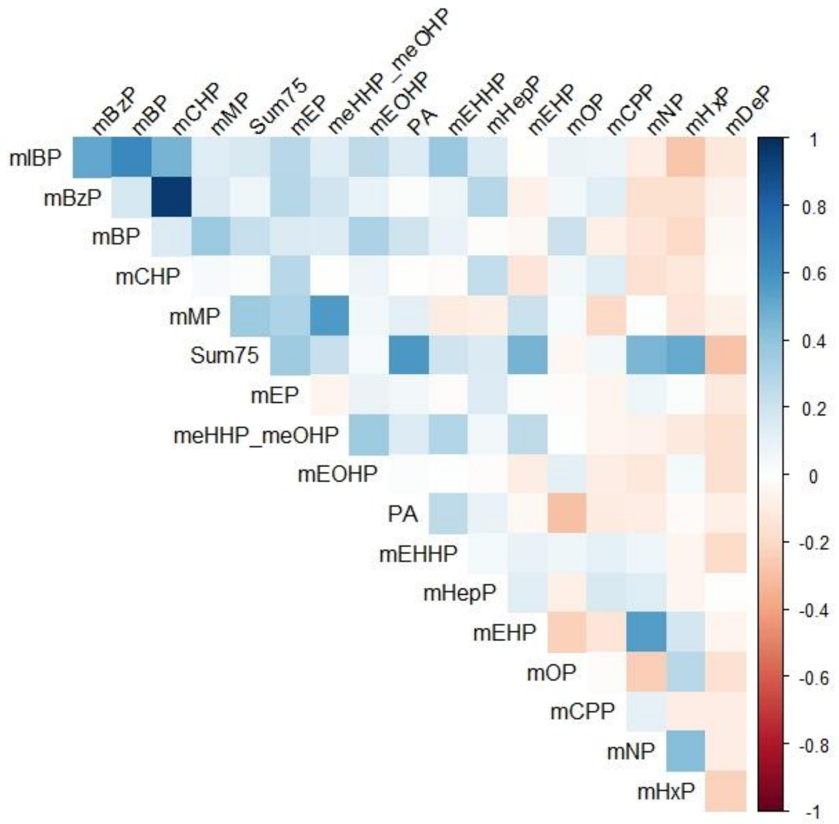


Figure 5.3. Correlations between phthalate metabolites found in the samples of loggerhead turtles from the western Mediterranean.

Concentrations of phthalate metabolites and their relationship with biometric and spatio-temporal variables

The location, origin, size and sex of the turtles, did not significantly influence the profile or concentrations of the metabolites in liver. This finding agrees with previous studies on marine mammals (García-Garín et al. 2022; Dziobak et al. 2021) and European eels (Forgous et al. 2016). However, a slight negative correlation was found between Σ_{12}PhMet (mMP, PA, mEP, mBP, mHxP, mNP, mHepP, mEHP, mEHHP, mCPP, mIBP, and mOP) concentration and size (CCL, r = -0.31). This correlation indicated that the larger the size of the turtle, the lower the concentration of those metabolites. The same was observed with the ΣmeHHP+meOHP concentration and size (r = -0.298). A negative correlation between the harbour porpoises' size (*Phocoena phocoena*) and PA was previously observed (Rian et al., 2020). Previous studies indicated that contaminants, including phthalates and their metabolites, tend to be more concentrated in smaller individuals (Mckenzie et al. 1999; Rian et al. 2020); although other studies show no relationship between contaminant concentrations and size in marine turtles and other species, including cetaceans and fish (Fourgous et al. 2016; Guerranti et al. 2016; Novillo et al. 2017; Savoca et al. 2018; Page-Karjian et al. 2020; Rian et al. 2020; Dziobak et al. 2021). Similarly, no correlations were found between the concentration of phthalate metabolites and the sex of the analyzed turtles. However, more research is needed concerning these variables, as this is the first study to explore a possible relationship between phthalates metabolites and sex.

Phthalate metabolites concentrations in samples from 2021 differed significantly from the rest of the sampling years ($p = 0.0005$). The Σ_{12}PhMet concentration was increasing steadily since 2016 and a steeper increase in 2021 (figure 5.4, median in 2022: 258.1 ng/g d.w.) than in previous years (figure 5.4, median in 2016: 112.2 ng/g d.w.; 2017: 67.6 ng/g d.w.; 2018: 146.2 ng/g d.w.; 2019: 96.4 ng/g d.w.; and in 2020: 163.9 ng/g d.w.) was observed.

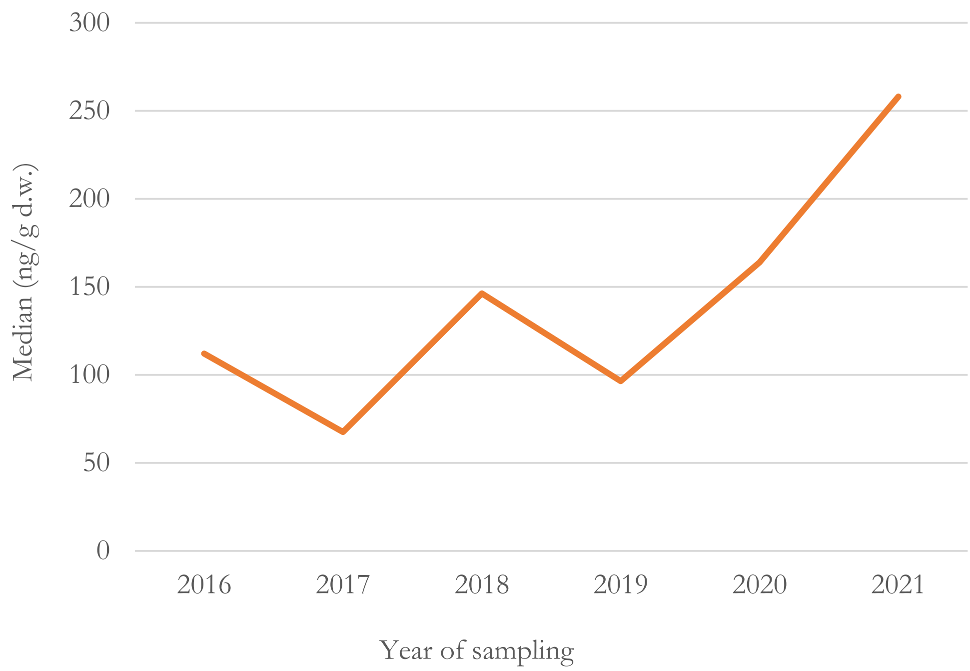


Figure 5.4. Evolution of Σ_{12}PhMet concentrations in the years of sampling loggerhead turtles fron the western Mediterranean Sea (2016 - 2021).

This increase (figure 5.4) could be explained by an abnormally high discharge of waters coming from the surrounding agricultural fields and wastewater treatment plants (WWTPs) during 2020 and 2021. Several beaches in the area were repeatedly closed to the public due to unknown polluted water discharges (Cadena Ser, 2020; El Mundo Castellón, 2020; Rico J.A., 2020).

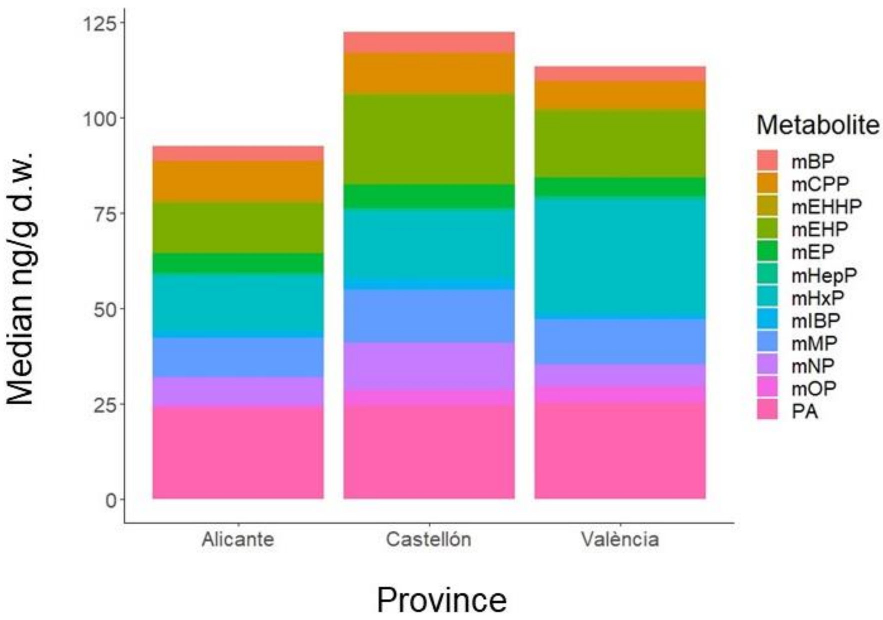


Figure 5.5. Sum concentrations (Σ_{12}PhMet) of the twelve most prevalent phthalate metabolites in liver samples of fresh carcasses of loggerhead turtles stranded or bycaught along the Valencian Community coast (E Spain; 15 individuals from Alicante, 41 individuals from Castellón and 23 individuals from València).

Nevertheless, it is known that phthalates do appear frequently in this type of water discharges in Spain (Céspedes et al. 2006) and that the Valencian Community is the second region in Spain with the highest volume of urban wastewater discharges (Ministerio de Agricultura, Pesca y Alimentación, 2022). Another possible explanation could be the constant increase in the amount of waste reaching the ocean, which is expected to continue (Jambeck et al. 2015; Borrelle et al. 2020), but it may also be the result of the increase of single-use plastic formulations and the mismanagement of plastic disposal following the Sars-CoV-2 pandemic (Peng et al. 2021). It is noteworthy that the Σ_{12}PhMet concentrations were found in higher concentrations in the more industrialised and populated province (figure 5.5), Valencia, although differences were not significant among provinces. Relative concentration of metabolites was similar in all the three provinces, which indicates a similar metabolic profile regardless of the origin of the turtles' report. Concentrations of phthalates metabolites and other contaminants are not necessarily associated; and therefore, the concentration of a contaminant in one environmental or biological compartment does not necessary allow to assess the risks in biota (Schmidt et al. 2021). Nowadays, is not possible to associate specific metabolites to specific sources, meaning that while loggerhead turtles constitute a good confirmation of the widespread presence of these pollutants, it is still necessary to properly identify sources and how they are distribured in the environment. It is also necessary to continue monitoring these chemicals in a variety of matrices to better understand risks and consequences of exposure.

Conclusions

Results indicate a high prevalence of phthalate metabolites in in loggerhead turtles' liver, therefore indicating exposure to plastics in the Mediterranean Sea. Concentrations found herein are generally lower to other studies with Mediterranean *Caretta caretta* and of the same order of magnitude than in other marine species and locations. These differences could be explained by differences in PAEs metabolism, by different exposure sources, and by a bigger sample size of the study. Biometric parameters, location of the samples and the origin of the turtles (stranded or bycaught) do not significantly influence phthalate metabolites concentration in this study. Regarding temporal analyses, an increase in phthalate metabolites' concentration was documented, especially during 2020.

Table S5.1: Compound name, compound abbreviation (abbr.), molecular formula, molecular weight (mol w.), MRM transitions for phthalate metabolites and their specific parameters used for the tandem MS determination of metabolites in marine turtles' livers.

Full name	Abbr.	Formula	Mol w.	CV (V)	CE (V)	Transitions
Monomethyl phthalate	mMP	$C_9H_8O_4$	180.2	8	20	178.9→77.1
				8	10	178.9→107.1
Monoethyl phthalate	mEP	$C_{10}H_{10}O_4$	194.2	36	18	193.0→77.1
				36	10	193.0→121.1
Mono-n-butyl phthalate	mBP	$C_{12}H_{14}O_4$	222.2	28	18	220.9→77.1
				28	12	220.9→149.1
Monoisobutyl phthalate	mIBP	$C_{12}H_{14}O_4$	222.2	10	16	220.9→77.1
				10	14	220.9→134.1
Mono(3-carboxypropyl) phthalate	mcPP	$C_{12}H_{12}O_6$	252.2	40	30	251.1→103
				40	18	250.9→121.1
Mono-n-pentyl phthalate	mPeP	$C_{13}H_{16}O_4$	236.3	10	18	235.0→77.1
				10	18	235.0→121.0
Monoisopentyl phthalate	mIPeP	$C_{13}H_{16}O_4$	235.3	10	18	235.1→77.1
				10	14	235.1→85.1
Mono-n-hexyl phthalate	mHxP	$C_{14}H_{18}O_4$	250.3	10	16	249.1→99.1
				10	14	249.1→99.1
Monocyclohexyl phthalate	mCHP	$C_{14}H_{16}O_4$	248.3	10	18	247.1→77.1
				10	18	247.1→97.1
Mono-n-heptyl phthalate	mHeP	$C_{15}H_{20}O_4$	264.3	10	20	263.0→77.1
				10	16	263.0→113.1
Monoebenzyl phthalate	mBzP	$C_{15}H_{12}O_4$	256.3	8	18	255.2→77.1
				8	20	255.2→183.0
Mono-n-octyl phthalate	mOP	$C_{16}H_{22}O_4$	278.3	10	18	277.1→77.1
				10	14	277.1→127.2

Table S5.1. (Continued). Compound name, compound abbreviation (abbr.), molecular formula, molecular weight (mol w.), MRM transitions for phthalate metabolites and their specific parameters used for the tandem MS determination of metabolites in marine turtles' livers.

Full name	Abbr.	Formula	Mol w.	CV (V)	CE (V)	Transitions
Mono(2-ethyl-1-hexyl) phthalate	mEHP	$C_{16}H_{22}O_4$	278.3	14	22	277.1→77.1
				14	14	277.1→134.1
Mono(2-ethyl-5-oxohexyl) phthalate	mEOHP	$C_{16}H_{20}O_5$	292.3	10	18	291.1→121.0
				10	14	291.1→143.1
Mono(2-ethyl-5-hydroxyhexyl) phthalate	mEHHP	$C_{16}H_{22}O_5$	294.4	12	20	293.1→121.0
				12	12	293.1→145.2
Mono-n-nonyl phthalate	mNp	$C_{17}H_{24}O_4$	292.4	10	24	291.1→77.1
				10	22	291.1→141.1
Mono-n-decyl phthalate	mDeP	$C_{18}H_{26}O_4$	306.4	12	20	305.1→77.1
				12	14	305.1→261.2
Phthalic acid	PA	$C_8H_6O_4$	166.1	6	14	165.0→77.1
				6	15	165.0→121.5
Internal Standards						
mono-Ethyl phthalate-3,4,5,6-d_4	mEP-d_4	$C_{10}H_6D_4O_4$	198.2	36	18	197.0→81.1
				36	10	197.0→125.1
mono-n-Butyl phthalate-3,4,5,6-d_4	mBP-d_4	$C_{12}H_{10}D_4O_4$	226.3	30	18	225.0→71.1
				30	10	225.0→181.1
mono-n-Nonyl phthalate-3,4,5,6-d_4	mNP-d_4	$C_{17}H_{20}D_4O_4$	296.4	44	20	295.1→81.1
				44	16	295.1→141.1

CV : cone voltage, CE: collision energy

Table S5.2: Retention time (RT), relative retention time (RRT), linear range, linearity, method limit of detection (mLOD) and method limit of quantification (mLOQ) for each target analyte in the UHPLC-MS/MS instrument.

Target analyte	RT# (min)	RRT (IS) ##	Linear range (ng/mL)	Linearity (R^2)	mLOD (ng/g d.w)	mLOQ (ng/g d.w)
mMP	3.22	0.72 (mEP-d_4)	0.50 - 50.0	1.00	0.83	2.50
mEP	4.55	1.01 (mEP-d_4)	0.50 - 50.0	0.999	0.83	2.50
mIBP	6.94	0.99 (mBP-d_4)	0.20 - 50.0	0.999	0.33	1.00
mBP	7.01	1.00 (mBP-d_4)	0.50 - 50.0	0.999	0.83	2.50
mPeP	7.93	1.76 (mEP-d_4)	0.50 - 50.0	0.998	0.83	2.50
mIPeP	7.78	1.73 (mEP-d_4)	0.50 - 50.0	0.999	0.83	2.50
mCHP	7.67	1.70 (mEP-d_4)	0.50 - 50.0	0.999	0.83	2.50
mHxP	8.54	1.90 (mEP-d_4)	0.50 - 50.0	0.999	0.83	2.50
mBzP	7.25	1.61 (mEP-d_4)	1.00 - 50.0	0.999	1.67	5.00
mHepP	8.73	1.94 (mEP-d_4)	0.50 - 50.0	1.00	0.83	2.50
mEHP	8.84	1.96 (mEP-d_4)	0.50 - 50.0	0.999	0.83	2.50
mOP	8.9	1.98 (mEP-d_4)	0.50 - 50.0	1.00	0.83	2.50
mEOHP	6.98	1.55 (mEP-d_4)	0.50 - 50.0	1.00	0.83	2.50
mEHHP	6.86	1.52 (mEP-d_4)	0.50 - 50.0	1.00	0.83	2.50
mCPP	3.21	0.71 (mEP-d_4)	2.00 - 50.0	0.961	3.33	10.0
mNP	9.09	1.00 (mNP-d_4)	0.50 - 50.0	0.999	0.83	2.50
mDeP	9.3	1.02 (mNP-d_4)	0.02 - 50.0	1.00	0.03	0.10
PA	2.3	0.51 (mEP-d_4)	10.0 - 50.0	0.943	16.6	50.0

#Average RT from matrix spiked standards (N=5).

##In parenthesis the internal standard (IS) used for quantification of the respective target analyte is shown.

Table S5.3: Absolute and relative recoveries (% ± RSD %, N=5, 20 ng), matrix effects (%), repeatability (RSD%, N=5, 20 ng) and ion ratios (% ± RSD%, N=5, 20 ng)

Target analytes	Absolute recovery (± RSD%)	Relative recovery (± RSD%)	Matrix effect (%) #	Repeatability RSD%	Ion Ratios % (± RSD%)
mMP	88.7 (± 10.0)	123 (± 3.41)	-22.5	2.37	84.5 (± 6.59)
mEP	91.1 (± 15.4)	73.4 (± 6.09)	65.3	1.02	52.9 (± 2.17)
mIBP	86.2 (± 5.78)	136 (± 2.07)	19.1	7.54	57.5 (± 3.86)
mBP	84.5 (± 6.15)	132 (± 2.65)	6.13	6.52	15.2 (± 5.06)
mPeP	66.4 (± 9.83)	97.9 (± 5.25)	45.2	10.4	14.5 (± 4.34)
mIPeP	68.5 (± 7.45)	101 (± 3.57)	21.6	7.53	58.6 (± 2.09)
mCHP	67.5 (± 7.91)	99.4 (± 1.77)	23.1	9.94	65.3 (± 2.18)
mHxP	50.0 (± 6.60)	63.9 (± 8.91)	39.2	3.83	46.2 (± 3.46)
mBzP	69.8 (± 16.3)	102.3 (± 15.1)	-3.86	9.69	15.5 (± 5.67)
mHepP	26.4 (± 11.8)	38.8 (± 14.1)	116	3.65	87.7 (± 4.57)
mEHP	26.8 (± 16.3)	29.5 (± 16.0)	-32.6	10.2	39.4 (± 1.99)
mOP	14.6 (± 19.2)	21.5 (± 22.3)	-29.1	9.20	76.7 (± 2.23)
mEOHP	85.0 (± 8.21)	125 (± 1.98)	5.53	5.95	96.7 (± 1.96)
mEHHP	82.3 (± 11.9)	121 (± 5.54)	1.79	6.44	98.3 (± 4.18)
mCPP	72.8 (± 32.4)	109 (± 28.7)	-33.3	10.7	NA
mNP*	9.77 (± 15.4)	168 (± 22.3)	42.3	0.89	101 (± 12.9)
mDeP*	5.53 (± 32.3)	98.3 (± 23.3)	3.19	0.88	47.5 (± 25.8)
PA*	103 (± 141)	8.51 (± 2.62)	38.2	2.46	11.7 (± 9.51)

*Semi-quantified due to low recoveries and/or high uncertainty.

#Calculated from fortification amount of 20 ng.

NA. Not applicable as only one transition was identified.

Table S5.4. Phthalate metabolites and their parent compounds.

Parent phthalate	Abbreviation	Phthalate metabolite	Abbreviation
Dimethyl phthalate	DMP	Monomethyl phthalate	mMP
Diethyl phthalate	DEP	Monoethyl phthalate	mEP
Di-n-butyl phthalate	DBP	Mono-n-butyl phthalate	mBP
Di-iso-butyl phthalate	DIBP	Mono-iso-butyl phthalate	mIBP
Di-n-pentyl phthalate	DnPeP	Mono-n-pentyl phthalate	mPeP
Di-iso-pentyl phthalate	DIPeP	Mono-iso-pentyl phthalate	mIPeP
Di-n-hexyl phthalate	DHxP	Mono-n-hexyl phthalate	mHxP
Di-n-heptyl phthalate	DHpP	Mono-n-heptyl phthalate	mHepP
Dicyclohexyl phthalate	DCHP	Monocyclohexyl phthalate	mCHP
Benzyl butyl phthalate	BBzP	Monobenzyl phthalate	mBzP
		Mono-n-butyl phthalate	mBP
Di-n-octyl phthalate	DnOP	Mono-n-octyl phthalate	mOP
		Mono(3-carboxypropyl) phthalate	mCPP
Di-n-nonyl phthalate	DnNP	Mono-n-nonyl phthalate	mNP
Di-n-decyl phthalate	DnDP	Mono-n-decyl phthalate	mDeP
Di(2-ethyl-1-hexyl) phthalate	DEHP	Mono(2-ethyl-1-hexyl) phthalate	mEHP
		Mono(2-ethyl-5-oxohexyl) phthalate	mEOHP
		Mono(2-ethyl-5-hydroxyhexyl) phthalate	mEHHP
		Phthalic acid*	PA

*Phthalic acid is a generic metabolite, therefore, no specific parent compound is indicated.

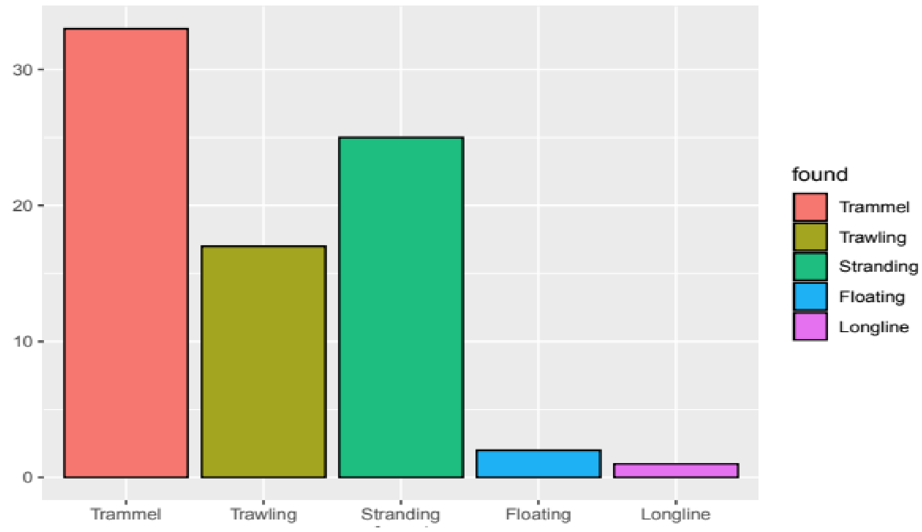


Figure S5.1. Bar plot showing the different origins of the loggerhead turtles from the western Mediterranean; the liver of which was analysed in this study.

Province	Municipality (north to south)	Number of turtles
Castellón	Benicarló	9
Castellón	Vinaròs	18
Castellón	Penyíscola	10
Castellón	Alcossebre	1
Castellón	Borriana	3
València	Port de Sagunt	1
València	València	2
València	El Saler	4
València	El Perelló	1
València	Cullera	1
València	Sueca	1
València	Tavernes de la Valldigna	2
València	Xeraco	1
València	Gandia	8
València	Oliva	2
Alicante	Dènia	1
Alicante	Benissa	1
Alicante	Calp	1
Alicante	Altea	3
Alicante	La Vilajoiosa	1
Alicante	Alicante	4
Alicante	Guardamar	1
Alicante	Torrevieja	1
Alicante	Santa Pola	1
Alicante	Pilar de la Horadada	1

Figure S5.2. Political map showing the different amount of turtles sampled in each province and classifying the municipalities by province in the Valencian Community.

References

Abalo-Morla, S., Marco, A., Tomás, J., Revuelta, O., Abella, E., Marco, V., ... & Belda, E. J., 2018. Survival and dispersal routes of head-started loggerhead sea turtle (*Caretta caretta*) post-hatchlings in the Mediterranean Sea. *Marine Biology*, 165(3), 1-17. https://doi.org/10.1007/s00227-018-3306-2

Asimakopoulos, A. G., Xue, J., De Carvalho, B. P., Iyer, A., Abualnaja, K. O., Yaghmoor, S. S., ... & Kannan, K., 2016. Urinary biomarkers of exposure to 57 xenobiotics and its association with oxidative stress in a population in Jeddah, Saudi Arabia. *Environmental Research*, 150, 573-581. https://doi.org/10.1016/j.envres.2015.11.029

Avio, C. G., Gorbi, S., and Regoli, F., 2017. Plastics and microplastics in the oceans: from emerging pollutants to emerged threat. *Marine Environmental Research*, 128, 2-11. https://doi.org/10.1016/j.marenvres.2016.05.012

Bai, R., Ma, F., Liang, D., & Zhao, X., 2009. Phthalic acid induces oxidative stress and alters the activity of some antioxidant enzymes in roots of Malus prunifolia. *Journal of Chemical Ecology*, 35(4), 488-494. https://doi.org/10.1007/s10886-009-9615-7

Baini, M., Martellini, T., Cincinelli, A., Campani, T., Minutoli, R., Panti, C., ... & Fossi, M. C., 2017. First detection of seven phthalate esters (PAEs) as plastic tracers in superficial neustonic/planktonic samples and cetacean blubber. *Analytical Methods*, 9(9), 1512-1520. http://doi.org/10.1039/C6AY02674E

Blair, J. D., Ikonomou, M. G., Kelly, B. C., Surridge, B., & Gobas, F. A., 2009. Ultra-trace determination of phthalate ester metabolites in seawater, sediments, and biota from an urbanized marine inlet by LC/ESI-MS/MS. *Environmental Science & Technology*, 43(16), 6262-6268. https://doi.org/10.1021/es9013135

Blasi, M. F., Avino, P., Notardonato, I., Di Fiore, C., Mattei, D., Gauger, M. F. W., ... and Favero, G., 2022. Phthalate esters (PAEs) concentration pattern reflects dietary habitats (δ13C) in blood of Mediterranean loggerhead turtles (*Caretta caretta*). *Ecotoxicology and Environmental Safety*, 239, 113619. https://doi.org/10.1016/j.ecoenv.2022.113619

Bjorndal, K. A., & Jackson, J. B., 2002. 10 roles of sea turtles in marine ecosystems: reconstructing the past. *The Biology of Sea Turtles*, 2, 259. ISBN: 1420040804.

Bonanno, G., & Orlando-Bonaca, M., 2018. Perspectives on using marine species as bioindicators of plastic pollution. *Marine Pollution Bulletin*, 137, 209-221. https://doi.org/10.1016/j.marpolbul.2018.10.018

Borrelle, S. B., Ringma, J., Law, K. L., Monnahan, C. C., Lebreton, L., McGivern, A., ... & Rochman, C. M., 2020. Predicted growth in plastic waste exceeds efforts to mitigate plastic pollution. *Science*, 369(6510), 1515-1518. https://doi.org/ 10.1126/science.aba3656

Cadena Ser Editorial, 2020. La depuradora de Pinedo vertió aguas fecales al mar 61 veces en el último año, según el PP. *Cadena Ser*. Accessed on the 22nd December 2022, https://cadenaser.com/comunitat-valenciana/2022/08/05/rueda-de-prensa-tras-el-pleno-del-consell-radio-valencia/.

Casale, P. and Tucker, A. D., 2015. *Caretta caretta*. The IUCN Red List of Threatened Species 2015. http://dx.doi.org/10.2305/IUCN.UK.2017-2.RLTS.T3897A119333622.en

Céspedes, R., Lacorte, S., Ginebreda, A., & Barceló, D., 2006. Chemical monitoring and occurrence of alkylphenols, alkylphenol ethoxylates, alcohol ethoxylates, phthalates and benzothiazoles in sewage treatment plants and receiving waters along the Ter River basin (Catalonia, NE Spain). *Analytical and Bioanalytical Chemistry*, 385(6), 992-1000. https://doi.org/10.1007/s00216-006-0448-8

Clark, M.A., Worrell, M.B. and Pless, J.E., 1997. Postmortem Changes in Soft Tissues. In: Haglund, W.D. and Sorg, M.H., Eds., Forensic Taphonomy: The Postmortem Fate of Human Remains, *CRC Press*, Boca Raton, 156-164.

Cózar, A., Aliani, S., Basurko, O. C., Arias, M., Isobe, A., Topouzelis, K., ... & Morales-Caselles, C., 2021. Marine litter windrows: a strategic target to understand and manage the ocean plastic pollution. *Frontiers in Marine Science*, *8*, 98. https://doi.org/10.3389/fmars.2021.571796

Crespo, J. L., García-Párraga, D., Giménez, I., Rubio-Guerri, C., Melero, M., Sánchez-Vizcaíno, J. M., ... & Muñoz, M. J., 2013. Two cases of pseudohermaphroditism in loggerhead sea turtles *Caretta caretta*. *Diseases of Aquatic Organisms*, 105(3), 183-191. https://doi.org/10.3354/dao02622

Darmon, G., Miaud, C., Claro, F., Doremus, G., & Galgani, F., 2017. Risk assessment reveals high exposure of sea turtles to marine debris in French Mediterranean and metropolitan Atlantic waters. *Deep Sea Research Part II: Topical Studies in Oceanography*, *141*, 319-328. https://doi.org/10.1016/j.dsr2.2016.07.005

Darmon, G., Schulz, M., Matiddi, M., Loza, A. L., Tòmàs, J., Camedda, A., ... & Miaud, C., 2022. Drivers of litter ingestion by sea turtles: Three decades of empirical data collected in Atlantic Europe and the Mediterranean. *Marine Pollution Bulletin*, 185, 114364. https://doi.org/10.1016/j.marpolbul.2022.114364

Domènech, F., Aznar, F. J., Raga, J. A., & Tomás, J., 2019. Two decades of monitoring in marine debris ingestion in loggerhead sea turtle, *Caretta caretta*, from the western Mediterranean. *Environmental Pollution*, *244*, 367-378. https://doi.org/10.1016/j.envpol.2018.10.047

Domínguez-Romero, E., & Scheringer, M., 2019. A review of phthalate pharmacokinetics in human and rat: what factors drive phthalate distribution and partitioning? *Drug metabolism reviews*, 51(3), 314-329. https://doi.org/10.1080/03602532.2019.1620762

Dziobak, M. K., Wells, R. S., Pisarski, E. C., Wirth, E. F., & Hart, L. B., 2021. Demographic Assessment of Mono (2-ethylhexyl) Phthalate (MEHP) and Monoethyl Phthalate (MEP) Concentrations in Common Bottlenose Dolphins (*Tursiops truncatus*) From Sarasota Bay, FL, USA. *GeoHealth*, 5(5), e2020GH000348. https://doi.org/10.1029/2020GH000348

El Mundo Editorial, 2020. Burriana cierra al baño la playa de la Malvarrosa por contaminación de aguas fecales. *El Mundo Castellón*. Accessed on the 22nd December 2022. https://www.elmundo.es/comunidad-valenciana/castellon/2020/06/09/5edf92eafdddffef0b8b456f.html

Eriksen, M., Lebreton, L. C., Carson, H. S., Thiel, M., Moore, C. J., Borerro, J. C., ... and Reisser, J., 2014. Plastic pollution in the world's oceans: more than 5 trillion plastic pieces weighing over 250,000 tons afloat at sea. *PloS One*, 9(12), e111913. https://doi.org/10.1371/journal.pone.0111913

Fossi, M. C., Coppola, D., Baini, M., Giannetti, M., Guerranti, C., Marsili, L., ... & Clò, S., 2014. Large filter feeding marine organisms as indicators of microplastic in the pelagic environment: the case studies of the Mediterranean basking shark (*Cetorhinus maximus*) and fin whale (*Balaenoptera physalus*). *Marine environmental research*, *100*, 17-24. https://doi.org/10.1016/j.marenvres.2014.02.002

Fossi, M. C., Pedà, C., Compa, M., Tsangaris, C., Alomar, C., Claro, F., ... & Baini, M., 2018. Bioindicators for monitoring marine litter ingestion and its impacts on Mediterranean biodiversity. *Environmental Pollution*, 237, 1023-1040. https://doi.org/10.1016/j.envpol.2017.11.019

Frederiksen, H., Nielsen, O., Koch, H. M., Skakkebaek, N. E., Juul, A., Jørgensen, N., & Andersson, A. M., 2020. Changes in urinary excretion of phthalates, phthalate substitutes, bisphenols and other polychlorinated and phenolic substances in young Danish men; 2009–2017. *International journal of hygiene and environmental health*, 223(1), 93-105. https://doi.org/10.1016/j.ijheh.2019.10.002

Fromme, H., Bolte, G., Koch, H. M., Angerer, J., Boehmer, S., Drexler, H., ... & Liebl, B., 2007. Occurrence and daily variation of phthalate metabolites in the urine of an adult population. International journal of hygiene and environmental health, 210(1), 21-33. https://doi.org/10.1016/j.ijheh.2006.09.005

Fourgous, C., Chevreuil, M., Alliot, F., Amilhat, E., Faliex, E., Paris-Palacios, S., ... & Goutte, A., 2016. Phthalate metabolites in the European eel (*Anguilla anguilla*) from Mediterranean coastal lagoons. *Science of the Total Environment*, 569, 1053-1059. https://doi.org/10.1016/j.scitotenv.2016.06.159

Garcia-Garin, O., Sahyoun, W., Net, S., Vighi, M., Aguilar, A., Ouddane, B., ... & Borrell, A., 2022. Intrapopulation and temporal differences of phthalate concentrations in North Atlantic fin whales (*Balaenoptera physalus*). *Chemosphere*, 300, 134453. https://doi.org/10.1016/j.chemosphere.2022.134453

Geraci, J. R., & Lounsbury, V. J., 2005. Marine mammals ashore: a field guide for strandings. National Aquarium in Baltimore. ISBN:1883550017.

Gibbons, J. W., Scott, D. E., Ryan, T. J., Buhlmann, K. A., Tuberville, T. D., Metts, B. S., ... and Winne, C. T., 2000. The Global Decline of Reptiles, Déjà Vu Amphibians: Reptile species are declining on a global scale. Six significant threats to reptile populations are habitat loss and degradation, introduced invasive species, environmental pollution, disease, unsustainable use, and global climate change. *BioScience*, *50*(8), 653-666. https://doi.org/10.1641/0006-3568(2000)050[0653:TGDORD]2.0.CO;2

Guerranti, C., Cau, A., Renzi, M., Badini, S., Grazioli, E., Perra, G., & Focardi, S. E., 2016. Phthalates and perfluorinated alkylated substances in Atlantic bluefin tuna (*Thunnus thynnus*) specimens from Mediterranean Sea (Sardinia, Italy): Levels and risks for human consumption. *Journal of Environmental Science and Health, Part B*, *51*(10), 661-667. https://doi.org/10.1080/03601234.2016.1191886

Hannas, B. R., Lambright, C. S., Furr, J., Howdeshell, K. L., Wilson, V. S., & Gray Jr, L. E., 2011. Dose-response assessment of fetal testosterone production and gene expression levels in rat testes following in utero exposure to diethylhexyl phthalate, diisobutyl phthalate, diisoheptyl phthalate, and diisononyl phthalate. *Toxicological Sciences*, 123(1), 206-216. https://doi.org/10.1093/toxsci/kfr146

Hart, L. B., Beckingham, B., Wells, R. S., Alten Flagg, M., Wischusen, K., Moors, A., ... & Wirth, E., 2018. Urinary phthalate metabolites in common bottlenose dolphins (*Tursiops truncatus*) from Sarasota Bay, FL, USA. *GeoHealth*, *2*(10), 313-326. https://doi.org/10.1029/2018GH000146

Hlisníková, H., Petrovičová, I., Kolena, B., Šidlovská, M., & Sirotkin, A., 2020. Effects and mechanisms of phthalates' action on reproductive processes and reproductive health: a literature review. *International Journal of Environmental Research and Public Health*, 17(18), 6811. https://doi.org/10.3390/ijerph17186811

Hotchkiss, A. K., Parks-Saldutti, L. G., Ostby, J. S., Lambright, C., Furr, J., Vandenbergh, J. G., & Gray Jr, L. E., 2004. A mixture of the "antiandrogens" linuron and butyl benzyl phthalate alters sexual differentiation of the male rat in a cumulative fashion. *Biology of reproduction*, *71*(6), 1852-1861. https://doi.org/10.1095

Hu, X., Gu, Y., Huang, W., and Yin, D., 2016. Phthalate monoesters as markers of phthalate contamination in wild marine organisms. *Environmental pollution*, 218, 410-418. https://doi.org/10.1016/j.envpol.2016.07.020

INE 2021. [accessed 16th March 2022; https://www.ine.es/jaxiT3/Tabla.htm?t=2852]

Jambeck, J. R., Geyer, R., Wilcox, C., Siegler, T. R., Perryman, M., Andrady, A., ... & Law, K. L., 2015. Plastic waste inputs from land into the ocean. Science, 347(6223), 768-771. https://doi.org/10.1126/science.1260352

Kassotis, C. D., Tillitt, D. E., Lin, C. H., McElroy, J. A., & Nagel, S. C., 2016. Endocrine-disrupting chemicals and oil and natural gas operations: potential environmental contamination and recommendations to assess complex environmental mixtures. *Environmental health perspectives*, *124*(3), 256-264. https://doi.org/10.1289/ehp.1409535

Kluwe, W. M., 1982. Overview of phthalate ester pharmacokinetics in mammalian species. *Environmental Health Perspectives*, 45, 3-9. https://doi.org/10.1289/ehp.82453

Kumar, N., Srivastava, S., & Roy, P., 2015. Impact of low molecular weight phthalates in inducing reproductive malfunctions in male mice: Special emphasis on Sertoli cell functions. *General and Comparative Endocrinology*, *215*, 36-50. https://doi.org/10.1016/j.ygcen.2014.09.012

Lazar, B. and Gračan, R., 2011. Ingestion of marine debris by loggerhead sea turtles, *Caretta caretta*, in the Adriatic Sea. *Marine pollution bulletin*, *62*(1), 43-47.https://doi.org/10.1016/j.marpolbul.2010.09.013

Lynch, J. M., 2018. Quantities of marine debris ingested by sea turtles: global meta-analysis highlights need for standardized data reporting methods and reveals relative risk. *Environmental science & technology*, *52*(21), 12026-12038. https://doi.org/10.1021/acs.est.8b02848

Maffucci, F., Caurant, F., Bustamante, P., & Bentivegna, F., 2005. Trace element (Cd, Cu, Hg, Se, Zn) accumulation and tissue distribution in loggerhead turtles (*Caretta caretta*) from the Western Mediterranean Sea (southern Italy). *Chemosphere*, 58(5), 535-542. https://doi.org/10.1016/j.chemosphere.2004.09.032

Ministerio de Agricultura, Pesca y Alimentación (2022). Vertidos por volumen anual y población-equivalente. Accessed on the 22nd December 2022. https://sig.mapama.gob.es/WebServices/clientews/intranet/default.aspx?nombre=CNV_ESTADISTICA_4&claves=&valores=&origen=2.

McKenzie, C., Godley, B. J., Furness, R. W., & Wells, D. E., 1999. Concentrations and patterns of organochlorine contaminants in marine turtles from Mediterranean and Atlantic waters. *Marine Environmental Research*, *47*(2), 117-135. https://doi.org/10.1016/S0141-1136(98)00109-3

Mrosovsky, N., Kamel, S., Rees, A. F., & Margaritoulis, D., 2002. Pivotal temperature for loggerhead turtles (Caretta caretta) from Kyparissia Bay, Greece. *Canadian Journal of Zoology*, 80(12), 2118-2124. https://doi.org/10.1139/z02-204

Nelms, S. E., Duncan, E. M., Broderick, A. C., Galloway, T. S., Godfrey, M. H., Hamann, M., ... and Godley, B. J., 2016. Plastic and marine turtles: a review and call for research. *ICES Journal of Marine Science*, *73*(2), 165-181.

Net, S., Sempere, R., Delmont, A., Paluselli, A., and Ouddane, B., 2015. Occurrence, fate, behavior and ecotoxicological state of phthalates in different environmental matrices. *Environmental Science* & Technology, 49(7), 4019-4035. https://doi.org/10.1021/es505233b

Novillo, O., Pertusa, J. F., and Tomás, J., 2017. Exploring the presence of pollutants at sea: monitoring heavy metals and pesticides in loggerhead turtles (*Caretta caretta*) from the western Mediterranean. *Science of the Total Environment*, *598*, 1130-1139. https://doi.org/10.1016/j.scitotenv.2017.04.090

Oehlmann, J., Schulte-Oehlmann, U., Kloas, W., Jagnytsch, O., Lutz, I., Kusk, K. O., ... & Tyler, C. R., 2009. A critical analysis of the biological impacts of plasticizers on wildlife. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 364(1526), 2047-2062. https://doi.org/10.1098/rstb.2008.0242

Otton, S. V., Sura, S., Blair, J., Ikonomou, M. G., & Gobas, F. A., 2008. Biodegradation of mono-alkyl phthalate esters in natural sediments. *Chemosphere*, 71(11), 2011-2016. https://doi.org/10.1016/j.chemosphere.2008.01.059

Page-Karjian, A., Lo, C. F., Ritchie, B., Harms, C. A., Rotstein, D. S., Han, S., ... & Perrault, J. R., 2020. Anthropogenic contaminants and histopathological findings in stranded cetaceans in the southeastern United States, 2012–2018. *Frontiers in Marine Science*, 7, 630. https://doi.org/10.3389/fmars.2020.00630

Paluselli, A., Fauvelle, V., Schmidt, N., Galgani, F., Net, S., & Sempere, R., 2018a. Distribution of phthalates in Marseille bay (NW Mediterranean Sea). *Science of the total environment*, *621*, 578-587. https://doi.org/10.1016/j.scitotenv.2017.11.306

Paluselli, A., Aminot, Y., Galgani, F., Net, S., & Sempere, R., 2018b. Occurrence of phthalate acid esters (PAEs) in the northwestern Mediterranean Sea and the Rhone River. *Progress in Oceanography*, *163*, 221-231. https://doi.org/10.1016/j.pocean.2017.06.002

Pan, G., Hanaoka, T., Yoshimura, M., Zhang, S., Wang, P., Tsukino, H., ... & Takahashi, K., 2006. Decreased serum free testosterone in workers exposed to high levels of di-n-butyl phthalate (DBP) and di-2-ethylhexyl phthalate (DEHP): a cross-sectional study in China. *Environmental health perspectives*, *114*(11), 1643-1648. https://doi.org/10.1289/ehp.9016

Peng, Y., Wu, P., Schartup, A. T., & Zhang, Y., 2021. Plastic waste release caused by COVID-19 and its fate in the global ocean. *Proceedings of the National Academy of Sciences*, 118(47). https://doi.org/10.1073/pnas.2111530118

R Core Team, 2021. R: A language and environment for statistical computing. *R Foundation for Statistical Computing*, Vienna, Austria. https://www.R-project.org/

Rocha, B. A., Asimakopoulos, A. G., Barbosa Jr, F., & Kannan, K., 2017. Urinary concentrations of 25 phthalate metabolites in Brazilian children and their association with oxidative DNA damage. *Science of the Total Environment*, *586*, 152-162. https://doi.org/10.1016/j.scitotenv.2017.01.193

Rian, M. B., Vike-Jonas, K., Gonzalez, S. V., Ciesielski, T. M., Venkatraman, V., Lindstrøm, U., ... & Asimakopoulos, A. G., 2020. Phthalate metabolites in harbor porpoises (*Phocoena phocoena*) from Norwegian coastal waters. *Environment international*, 137, 105525. https://doi.org/10.1016/j.envint.2020.105525

Rico, J. A., 2020. El Campello Cierra también Cala Palmeretes al confirmar la analítica otro vertido de fecales. *Información*. Accessed on 22nd December 2022. https://www.informacion.es/alacanti/2020/07/17/campello-cierra-cala-palmeretes-confirmar-8730874.html.

Rocha, B. A., Asimakopoulos, A. G., Barbosa Jr, F., & Kannan, K., 2017. Urinary concentrations of 25 phthalate metabolites in Brazilian children and their association with oxidative DNA damage. *Science of the Total Environmen*t, 586, 152-162. https://doi.org/10.1016/j.scitotenv.2017.01.193

Savoca, D., Arculeo, M., Barreca, S., Buscemi, S., Caracappa, S., Gentile, A., ... & Pace, A., 2018. Chasing phthalates in tissues of marine turtles from the Mediterranean Sea. *Marine pollution bulletin*, *127*, 165-169. https://doi.org/10.1016/j.marpolbul.2017.11.069

Savoca, D., Arculeo, M., Vecchioni, L., Cambera, I., Visconti, G., Melfi, R., ... & Pace, A., 2021. Can phthalates move into the eggs of the loggerhead sea turtle *Caretta caretta*? The case of the nests on the Linosa Island in the Mediterranean Sea. *Marine Pollution Bulletin*, 168, 112395. https://doi.org/10.1016/j.marpolbul.2021.112395

Schmidt, N., Castro-Jiménez, J., Oursel, B., & Sempéré, R., 2021. Phthalates and organophosphate esters in surface water, sediments and zooplankton of the NW Mediterranean Sea: Exploring links with microplastic abundance and accumulation in the marine food web. *Environmental Pollution*, 272, 115970. https://doi.org/10.1016/j.envpol.2020.115970

Sohn, J., Kim, S., Koschorreck, J., Kho, Y., and Choi, K., 2016. Alteration of sex hormone levels and steroidogenic pathway by several low molecular weight phthalates and their metabolites in male zebrafish (*Danio rerio*) and/or human adrenal cell (H295R) line. *Journal of hazardous materials*, 320, 45-54. https://doi.org/10.1016/j.jhazmat.2016.08.008

Song, Y., Hauser, R., Hu, F. B., Franke, A. A., Liu, S., & Sun, Q. (2014). Urinary concentrations of bisphenol A and phthalate metabolites and weight change: a prospective investigation in US women. *International journal of obesity*, 38(12), 1532-1537. https://doi.org/10.1038/ijo.2014.63

Standora, E. A., & Spotila, J. R., 1985. Temperature dependent sex determination in sea turtles. *Copeia*, 711-722. https://doi.org/10.2307/1444765

Suzuki, T., Yaguchi, K., Suzuki, S., & Suga, T., 2001. Monitoring of phthalic acid monoesters in river water by solid-phase extraction and GC-MS determination. *Environmental science & technology*, *35*(18), 3757-3763. https://doi.org/10.1021/es001860i

Swan, S. H., 2008. Environmental phthalate exposure in relation to reproductive outcomes and other health endpoints in humans. *Environmental research*, 108(2), 177-184. https://doi.org/10.1016/j.envres.2008.08.007

Tsochatzis, E. D., Tzimou-Tsitouridou, R., & Gika, H. G., 2017. Analytical methodologies for the assessment of phthalate exposure in humans. *Critical reviews in analytical chemistry*, 47(4), 279-297. https://doi.org/10.1080/19440049.2019.1615642

Weir, S. M., Talent, L. G., Anderson, T. A., & Salice, C. J., 2014. Unraveling the relative importance of oral and dermal contaminant exposure in reptiles: insights from studies using the western fence lizard (*Sceloporus occidentalis*). *PloS one*, *9*(6), e99666. https://doi.org/10.1371/journal.pone.0099666

Vorkamp, K., Dam, M., Riget, F., Fauser, P., Bossi, R., & Hansen, A. B., 2004. Screening of "new" contaminants in the marine environment of Greenland and the Faroe Islands. *NERI technical report*, (525).

Wickham H., 2016. ggplot2: Elegant Graphics for Data Analysis. *Springer-Verlag* New York. ISBN 978-3-319-24277-4, https://ggplot2.tidyverse.org

Zhang, H., Zhang, Z., Nakanishi, T., Wan, Y., Hiromori, Y., Nagase, H., & Hu, J. (2015). Structure-dependent activity of phthalate esters and phthalate monoesters binding to human constitutive androstane receptor. *Chemical Research in Toxicology*, 28(6), 1196-1204. https://doi.org/10.1021/acs.chemrestox.5b00028

6. Microdebris in three Spanish Mediterranean beaches located at a sporadic loggerhead turtles' (*Caretta caretta*) nesting area.

Published in: Novillo-Sanjuan, O., Raga, J. A., & Tomás, J. (2022). Microdebris in three Spanish Mediterranean beaches located at a sporadic loggerhead turtles'(*Caretta caretta*) nesting area. *Regional Studies in Marine Science*, 49, 102116. https://doi.org/10.1016/j.rsma.2021.102116

Abstract

We studied microdebris in three western Mediterranean beaches in East Spain. One of them is urban, while the other two are located in a protected environment. Moreover, one of them is used as hatchery for loggerhead turtles' (*Caretta caretta*) sporadic nesting activity. Here, we discuss the amount and type of microdebris in the area in different seasons, as well as at the surface and at 40 cm depth, were loggerhead turtles lay their nests. Total mean ± SD in July was 5.66 ± 3.66 MPs/kg at surface and 12.15 ± 7.76 MPs/kg at depth; while in November values were 6.45 ± 4.42 MPs/kg at surface and 5.51 ± 3.14 MPs/kg at depth. There were no significant differences among beaches, months, depths nor protection regime. Polymers found were, by descent order, polyethylene, rubber, latex, polypropylene and ethylene vinyl alcohol; which are mainly used in consumer goods, tires and food packaging. Overall, microdebris in these beaches are not among the highest in the Mediterranean and do not seem to threaten turtles' reproductive success, although more detailed studies are needed to determine potential effect on embryonic developmental processes.

Introduction

Plastic waste can be considered as the most evident human footprint of the Anthropocene. Only in Europe, 61.2 million tonnes of plastic were produced during 2019 (Plastics Europe, 2019). Plastics of different sizes, shapes and, origin end up in natural systems when not properly disposed, affecting the landscape and the organisms that inhabit in it. Plastics are now present in virtually every ecosystem on Earth. It has been calculated that from 4.8 to 12.7 million tons of plastic waste entered into the oceans in 2010 as a result of mismanaged urban waste from 192 coastal countries (Jambeck et al., 2015). Not only plastic input to the sea has been increasing ever since, but it is also predicted to be beyond the mitigation efforts (Borrelle et al., 2020). On top of that, plastic is even being considered as a planetary-boundary threat (Villarrubia-Gómez et al., 2018).

In line with the European Marine Strategy Framework Directive (MSFD), plastics measuring less than 5 mm are classified as microplastics (Galgani et al., 2013, Gago et al., 2016). Microplastics can be either directly manufactured to that size or smaller (primary microplastics) or they can originate from fragmentation of bigger plastic items that undergo physicochemical degradation when exposed to environmental conditions (secondary microplastics). Their distribution along the coasts may be influenced by many factors such as topology and geology, marine currents, weather, presence of river outflows, distance to cities and villages, tourism activity and other human interventions. Microplastics can reach the oceans due to both, terrestrial and marine activities, through direct spills or transported by running water, drainage systems, storm water, effluents form wastewater treatment plants (WWTP), sewers, landfills and other ways (Gregory and Andrady, 2011, Cole et al., 2011, Alomar et al., 2016, Murphy et al., 2016, Avio et al., 2017).

In the Mediterranean basin, tourism is the main factor contributing to marine litter input (Suaria and Aliani, 2014, Constant et al., 2019). There are approximately 350 million overnight stays per year, mostly during the summer months, which add to the already resident population of 160 million people (UNEP/MAP, 2012). This seasonal activity could account for differences in litter amounts during different times of the year, as already stated in prior studies (Bowman et al., 1998, Martinez-Ribes et al., 2007, Laglbauer et al., 2014; Munari et al. 2016; Pasternak et al., 2017, Prevenios et al., 2017, Vlachogianni et al., 2017). According to Prevenios et al. (2017), sea transport is the most influential variable in microplastic deposition at Mediterranean beaches, followed by in situ plastic dumping by beach users, wind, and runoff transport from inland; and big cities and river mouths are an important source of microplastics (Schmidt et al., 2018) On top of that, the Mediterranean Sea is an enclosed sea, which implies that it is prone to concentrate litter and contaminants due to its limited exchange of water through the Gibraltar Strait and the Suez Canal.

Beach sand pollution can be of special concern for certain protected species, such as loggerhead turtles, which is currently nesting sporadically in the western Mediterranean, in what seems an increasing trend as part of a potential colonization process (Casale and Tucker, 2017, Carreras et al., 2018) and references therein). Loggerhead turtles are oviparous reptiles that are born male or female according to temperature during the second third of the incubation period (Mrosovsky et al., 2002). Their pivotal temperature is around 29 °C. Above this temperature, the sex ratio is biased towards females and below this temperature towards males (Mrosovsky et al., 2002, Kaska et al., 2006, Wyneken and Lolavar, 2015). Moreover, incubation temperature also influences hatching's morphology, locomotor performance, response time to stimuli and, in general, survival (Fisher et al., 2014, Booth, 2017). Additionally, at temperatures above 33–35 °C and below 25 °C, hatching is not successful (Standora and Spotila, 1985, Gross et al., 1995). Hence, an overall change in surrounding sand temperature might result in sex biased or depleted loggerhead populations that can lead to collapse. Whether microplastics could be favouring this situation by increasing (Andrady, 2011, Yang et al., 2011, Beckwith and Fuentes, 2018) or decreasing temperature (Carson et al., 2011) is not clear. On top of that, microplastics could also decrease soil permeability, which could also be crucial to marine turtles' eggs development (Carson et al., 2011, Duncan et al., 2018). Also, microplastics can adsorb contaminants and trace metals from the environment; as well as leak their own additives posing a potential toxicological risk (Halle et al., 2020) for loggerhead turtles and their clutches.

In this context, the aims of this study are (1) to assess microplastic abundance and polymer types in three Spanish Mediterranean beaches, thus contributing to data recording in the Mediterranean region; (2) to ascertain whether there are seasonal differences in microplastic abundance and between urban and natural protected environments; and (3) to discuss potential implications of microdebris concentrations at surface and at depth in the incubation of loggerhead turtles' clutches.

Materials and methods

Studied area

We chose three fine sand beaches located in Valencia province (Spain): El Cabanyal, l'Alcatí and La Punta (figure 6. 2), the three of them facing the East. Sand in these beaches comes from fluvial and artificial sediments (Sanjaume and Pardo-Pascual, 2011). El Cabanyal beach (figure 6.2, b) is an urban beach located in the city of Valencia (800 215 inhabitants, INE, 2021). The beach includes recreational infrastructures and a promenade, and it is limited at the south by the Valencia port. There are three discharge waterways in the area, one north to the city of Valencia and two south to Valencia's port and the Turia river mouth (figure 2). As it is easily accessible by private and public transport is the one receiving the most visitors, particularly during the summer season (from June to September). Its sand is cleaned on a daily basis during summer, contrary to the other two locations. Cleaning in this beach, consists in raking over the first centimetres of sand with heavy machinery, removing big items from the surface. L'Alcatí beach is located in front of a luxury hotel and a golf course, although is protected by dunes (figure 2, c). Several local NGOs carry out manual clean-ups of macrolitter often. It does not receive as many visitors as El Cabanyal beach because it is located 20 km away from the city and public transport there is limited. Hence, this beach is mainly accessed by the hotel's clients and staff, or by people reaching the area either by private transport or walking long distances This beach is cleaned by hand mostly during summer months. Lastly, La Punta beach (figure 6.2, d) is located just south to l'Alcatí beach, forming part of the same stretch of coast, but its access is completely restricted due to its use as a recovery area for the endangered bird Kentish plover (*Charadrius alexandrines*). Although it is separated from L'Alcatí by a wood wall and it has an informative panel, few people do walk into it through the seashore. Moreover, it is usual to find great amounts of macrolitter because no cleaning is carried out. Finally, we should note that both L'Alcatí and La Punta beaches are located 11 km south to the Turia's river mouth and Valencia's port, inside the protected area of L'Albufera Natural Park (East Spain).

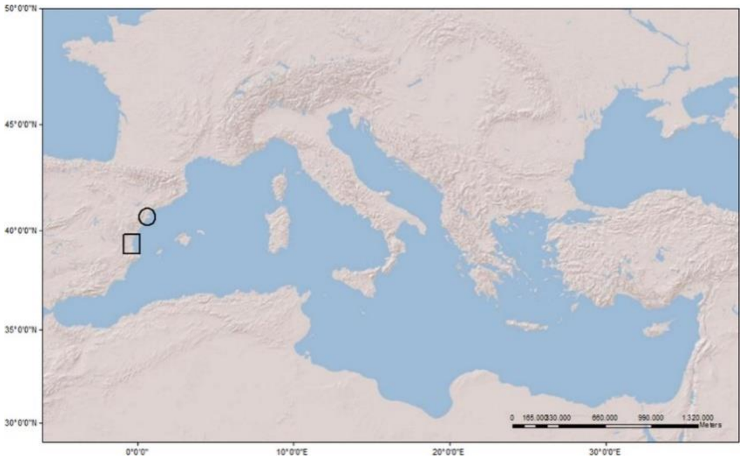


Figure 6.1. Map of the Mediterranean Sea. The area enclosed by a square constitutes the studied area, the area enclosed by a circle marks the Ebro River mouth.

The mouth of the Ebro river (one of the most abundant rivers in Spain) is located 180 km north from the beaches at the natural park and 165 km north from El Cabanyal beach (figure 6.1); also, main coastal sea currents flow from north to south (Millot, 1987, García-Ladona, 2017). All of these locations are under the influence of some WWTPs (Waste

Water Treatment Plants) and, to a less extent, under the influence of the Ebro River (figure 6.1), which is known to carry high amounts of microplastics, most of them fibres (Simon-Sánchez et al., 2019).

Figure 6.2. Close-ups of the studied area. (A) The gulf of Valencia. Red circles mark beach's location, the red arrow marks the Turia River mouth, and white arrows mark the location of WWTPs; (b) El Cabanyal beach, the red arrow marks the port; (c) L'Alcatí beach, the arrows mark the location of the hotel and its golf court; (d) La Punta Beach.

Sampling procedure

Two samplings were carried out in the three beaches, being the first one on July the 6th (2018), at the beginning of the summer season, and the second one on November the 6th (2018), after the summer season. Following the MSFD and national guidelines (Gago et al., 2016, Ministry of Agriculture and Fishing, 2017), the maximum microplastic size considered was 5 mm in its largest dimension and the smallest size category was <0.2 mm. Specifically, size categories were: <0.2 mm, 0.2–0.4 mm, 0.4–0.6 mm, 0.6–0.8 mm, 0.8–1 mm, 1–2 mm, 2–3 mm, 3–4 mm and 4–5 mm.

At each beach, 3 sample sites were randomly chosen in the high tide line, each site being separated by 50 m. Coordinates of the sampling sites are available in Supplementary Material, table 6.1. We chose the high tide line in accordance to most coastal microplastic studies and because there are not important tide variations in the Mediterranean; the high tide line only changes when there are storms. Additionally, loggerheads' nesting activity in the area is sporadic, so a specific nesting preference in these beaches cannot be established. At each sampling site, two samples were taken, one from the first centimetre of sand and another one at 40 cm depth, which is the average depth at which loggerhead turtles dig the nest chamber to lay eggs (Godley et al., 2001). Sand was stored in metallic containers with volumes of 2.5 L (n = 6 per beach, in total, n = 18) that were previously cleaned and inspected for microplastics. Sampling sites consisted of an area of 0.25 m^2, delimited by a 50 × 50 cm cord quadrat. A metallic shovel was used to dig and collect sand.

Laboratory procedure

We weighed sand samples and followed the optimal extraction parameters resulting by a study performed by Besley et al. (2017). Briefly, samples were dried in an oven at 50 °C for 48 h inside glass trays covered with perforated aluminium foil. A supersaturated filtered solution of NaCl was prepared with Milli-Q water (358.9 g/L) to perform a density

Table 6.1. Interpretation of the numeric result of the CCI_{mp} (Clean Coast Index for microplastics).

CCI_{mp}	**Cleanness regarding microplastics**
0–0.5	Very clean
0.5–1	Clean
1–2	Moderately dirty
2–3	Dirty
>3	Very dirty

separation. The solution was stirred in a magnetic stirrer at 600–700 rpm and 60 °C for periods of 8 h during three days. Once the solution was ready it was added to the sand samples, homogenized and left settling for at least 6 h. After settling, samples were filtered under vacuum with GC/F microfiber filters in a laminar flow cabinet in order to ensure that no impurities or contamination from the working environment were present. The supernatant was subjected to four consecutive filtrations in order to guarantee maximum microplastic recovery. Finally, all the filters were examined under a stereomicroscope (Leica MZ APO, 8–80x, figure 6.5) and microplastics were quantified and classified by shape and colour. Photographs of the microplastics and other items were taken with a macroscope Leica Z16/DFC500 coupled with a computer.

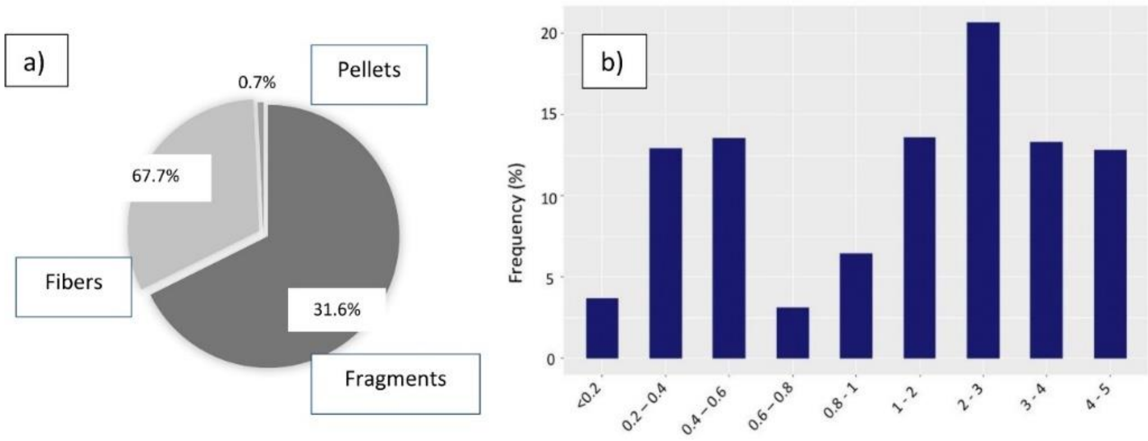


Figure 6.3. (a) Microplastics' type proportions and (b) microplastics' size distribution of the total collected items in the present study.

Polymer analyses

A subsample of 110 microdebris items were randomly separated for polymer analyses by FT-IR (Fourier Transformed Infrared Spectroscopy; Agilent Technology Cary 630 spectrometer), but 9 of them were not taken into account for the analyses because they were of natural origin. Of the remaining 101, 44 were from Cabanyal beach, 46 from L'Alcatí beach and 11 from La Punta beach. We gently rinsed these microplastics with deionized water in order to get rid of dirt that could interfere with the spectrometer scans (Jung et al., 2018).

For accuracy purposes, both the base of the spectrometer and the ATR diamond were thoroughly cleaned with propanol before and after procedure, between every sample and in between measurements of the same sample. For this same purpose, the background was scanned eight times before every sample, although in this case the background was just the air filling the working space because no sample inclusion in any material was required.

Measurements were carried out in ATR mode (Attenuated Total Reflection) and within a wavelength range of 4000–650 cm -1. The experimental nominal working resolution was set to 4 and the Happ–Genzel function was the chosen apodization. Each sample was scanned three times at different points of their surface when possible to ensure good spectra acquisition. However, when the samples were too brittle or too small only one point of the surface was pressed by the diamond tip.

Statistical analyses

Exploratory analyses were performed in RStudio (version 1.0.143; R. Core Team, 2019) and graphical representations of data were crafted using the "ggplot2" package (Wickham, 2009). Normality of the data was assessed graphically and through tests like Shapiro–Wilk. As data was not normally distributed, we used the U-Mann–Whitney test to account for analysing pair-wise differences, and Kruskal Wallis tests to analyse whether there were significant differences between beaches, depths and months. Resulting FTIR spectra were compared with those from the native Agilent library in the spectrometer's software, Agilent Microlab.

Clean Coast Index (CCI)

We calculated the Clean Coast Index proposed by Alkalay et al. (2007), both in July and November and just taking into account the presence of microplastics. The formula is as follows:

$$CCImp = \frac{MPs}{Area} x\ K$$

Where CCI is Clean Coast Index for microplastics, MPs is the average of microplastics found in the three sampling sites at each beach and K is a constant (0.02). We adapted "K" from 20 to 0.02 for convenience, following Fernandino et al. (2015) methodology. The reason is that CCI is calculated including macrodebris, and the results are expressed in a scale of 0–20 according to the amount of visible litter accumulating on sand. However, most of microlitter items are not visible by naked eye, therefore a visual identification would lead to unreal results. Instead, the results were interpreted following the criteria presented in table 6.1.

Contamination control

Only glass and stainless-steel material was used throughout the whole process in order to avoid contamination. White cotton coats and blue nitrile gloves were worn so as to identify potential contamination from clothes easily and remove it accordingly to prevent false positives. All surfaces and material were thoroughly cleaned with ethanol 70% before use. Petri dishes were previously inspected under a stereomicroscope.

Blank filters (GF/C) were left next to the real samples to prevent air-borne contamination during filtration and observation and Milli-Q water was filtered under vacuum prior to NaCl solution preparation.

Results

Amount and type of microdebris

All the samples collected presented microdebris, being the majority of items fibers (67.7%) and secondary microplastic fragments and other products of degradation from synthetic materials (31.6%). Only five items were primary microplastics (0.7%; figure 6.3, a). Of the last, two were black disks (ø = 4 mm) and three were white microbeads (ø = 3.5 mm). In total, 99.3% of the microplastics were of secondary origin. Among them, we could recognize some as food packaging, water bottle taps, paint, fishing lines, rubbers and glitter.

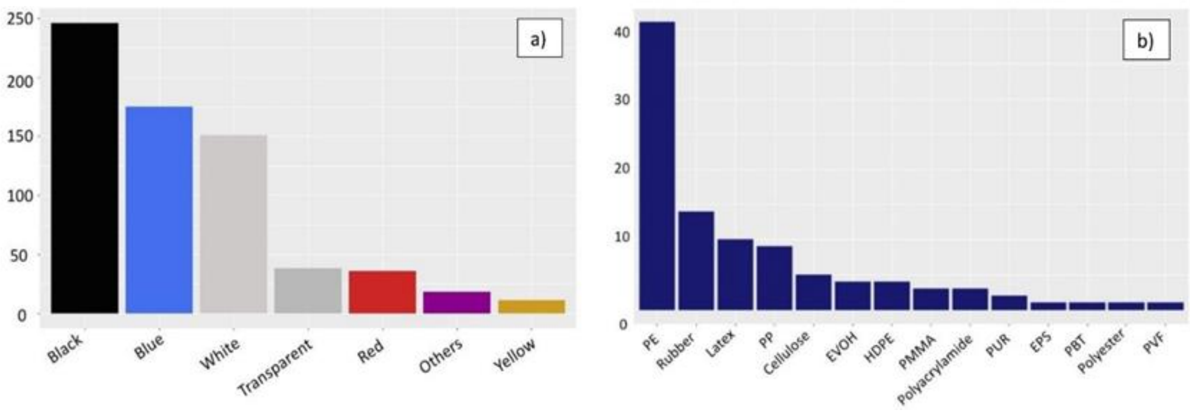


Figure 6.4. (a) Colour distribution of the anthropogenic microplastics found; (b) Polymer frequency resulting from the items analysed by FT-IR. PE: polyethylene, PP: polypropylene, EVOH: Ethylene vinyl alcohol, HDPE: high density polyethylene, PMMA: polymethyl methacrylate, PUR: Polyurethane, EPS: polystyrene, PBT: polybutyl terephthalate, PVF: polyvinyl

fluoride. “Rubber” includes: ethylene propylene diene (EPD), polyisobutylene (PIB), polybutadiene and polysulfide.

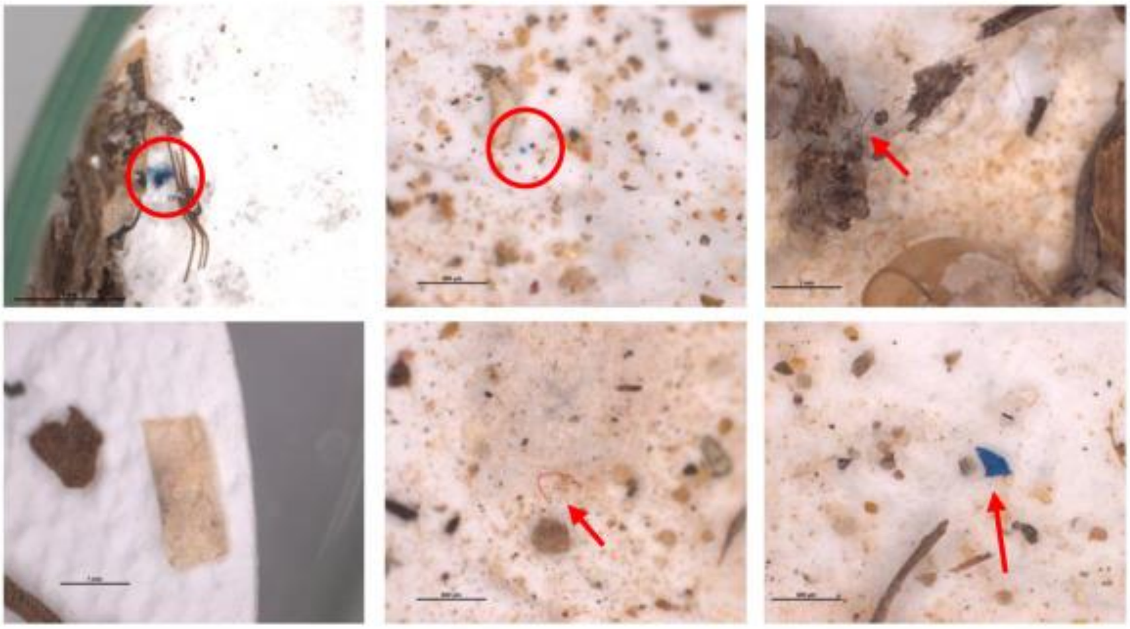


Figure 6.5. Microplastics found in the present study. Red circles and arrows mark de presence of a microplastic in the sample. Photographs were taken under a macroscope Leica Z16/DFC500.

Average microdebris per beach is showed in table 6.2. Amounts per sample ranged from 2 to 605 microplastics, including two outliers: one sample with 605 microplastics (La Punta) and another one with 305 microplastics (L'Alcatí). When including these outliers, mean SD at surface was 58.2 ± 13.55 MPs/kg and 66.64 ± 7.07 MPs/kg at depth; while in winter there were 39.77 ± 10.48 MPs/kg at surface and 37.40 ± 8.7 at depth. Lately, these outliers were removed due to their unreal influence in the analysis (both of them surpassed the counts in the rest of the sampling sites by at least 200 microplastics). When excluding these outliers, we counted 704 microplastics in total, 371 in July and 333 in November. In the summer, mean SD at surface was 5.66 ± 3.66 MPs/kg and 12.15 MPs/kg at depth; while in winter there were 6.45 ± 4.42 MPs/kg at surface and 5.51 ± 3.13 MPs/kg.

Regarding size distribution, most of the microplastics measured between 2 and 3 mm, followed by those measuring 0.4–0.6 mm, 1–2 mm and 3–4 mm (figure 6.3, b). The proportion of microplastics that measured less than 1 mm ranged between 43.6% up to 72.4%, with no significant differences regarding location and month.

Black was the most frequent color encountered (35.6%) followed by blue (25.4%) and white (21.9%). The frequency of the rest of the colors was far below. These colorswere transparent (5.5%), red (5.4%), yellow (1.5%) and others (2.3%) (figure 6.4, a).

Table 6.2. Mean number of microplastics/kg ± SD collected at each beach, depth, and month; and in all the beaches, depths, and months altogether.

Month	Depth	Cabanyal	L'Alcatí	La Punta
July	1 cm	9.74 ± 2.03	4.53 ± 4.03	2.69 ± 1.23
July	40 cm	19.24 ± 15.01	3.85 ± 1.78	13.34 ±7.08
November	1 cm	5.88 ± 3.45	11.13 ± 13.18	2.35 ± 0.93
November	40 cm	4.63 ± 3.27	8.99 ± 4.70	2.90 ± 3.60
November and July	Both	9.875 ± 6.14	7.12 ± 3.5	5.32 ± 5.53

Clean Coasts Index and differences between variables

CCI_{mp} in July showed that Cabanyal Beach (Valencia, urban beach) was dirty (2.62) and that L'Alcatí and La Punta beaches (in the natural park) were moderately dirty (1.04 and 1.67, respectively). In November, CCI_{mp} showed that El Cabanyal and La Punta beaches were clean (0.71 and 0.55, respectively) and that L'Alcatí beach was moderately dirty (1.82).

No significant differences were found between microdebris mean at depth and at surface, neither between means at the different beaches nor between means between months (table 6.2, $p > 0.005$ in all cases).

Polymer analyses results

In total, 101 items were analysed by FT-IR and 18 different polymers were identified by this technique. Not all of these materials were plastic polymers and 7 items were removed because the match did not arrive to 60%. Cellulose is showed but these items were removed from the aforementioned analyses. The most frequent polymer found was PE, followed by far by rubbers (EPD, PIB, polybutadiene and polysulfide), latex and PP (figure 6.3., b). The only item identified as PVF corresponded to a fishing line, and TiO2 corresponded to a piece of blue paint.

None of the rivers that could have influence on these beaches experimented any flood event before and between sampling days.

Discussion

Microplastics abundance and distribution

Microplastics were found in all three beaches and in every sample taken, which supports the idea that these pollutants are already ubiquitous in marine and coastal environments. Values seem low even though these beaches are under close influence of Valencia's WWTP, the Turia River, and, possibly, under the influence of the Ebro River as well, since dominant coastal current in the area flow north to south. If outliers are not taken into account, our data is amongst the lowest in the Mediterranean basin, together with those found in beaches in the Adriatic Sea (Munari et al., 2017) and in the Aegean Sea (Kaberi et al., 2013; table 6.3). If both outliers are taken into account, data in our study resembles values obtained also for the Aegean Sea (Kaberi et al., 2013, Piperagkas et al., 2019), but also in the Western Mediterranean Sea (Bayo et al., 2019; table 6.3). However, even including these extreme cases, the amounts in our study were amongst the lowest and, specifically, they were far below the high concentrations found in Cyprus by Duncan et al. (2018, table 6.3). This difference in litter accumulation could be explained by the little interaction of the Levantine Basin with the rest of the Mediterranean Sea, consequence of the weak north-eastward currents of the area (Hecht et al., 1988). This interaction is especially weak in the northern coast of Cyprus (Hetch et al. 1988; Alhammoud et al., 2005, Duncan et al., 2018). Lagrangian models further confirm this theory, suggesting that the Levantine Basin do tends to accumulate litter in the long-term (Lebreton et al., 2012, Sebille et al., 2015, Zambiachi et al., 2017, Liubarsteva et al., 2018, Mansui et al., 2020) and that western Mediterraenan coasts tend to be free of this phenomenon due to stronger currents that either tend to accumulate litter in specific areas or scatter it depending on the region (Mansui et al., 2015). Studying sea currents and winds could be the key to understand why there is differential deposition in

Table 6.3. Microplastics' studies at beaches in the Mediterranean Sea. In studies where many polymers were analysed, only the five most abundant are displayed.

	Location	**Surface/Depth**	**Concentration**	**Polymer**	**Shape**
Kaberi et al. (2013)	Aegean Sea	Surface	10–575 MPs/m^2	PE > PP > PET	Pellets
Bayo et al. (2019)	Western Mediterranean	Surface	53.1 ± 7.6 microlitter/kg	LDPE > HDPE > PVE > PP > PS	Fragments > **fibres** > film > pellets > foam
Laglbauer et al. (2014)	Adriatic Sea	Surface	Shoreline: 133.3 MPs/kg infralitoral: 155.6 MPs/kg	Not analysed	Fibres
Lots et al. (2017)	Several countries	Surface	76 ± 13 – 1512 ± 187 MPs/kg	Polyester > PE > PP	Fibres
Munari et al. (2017)	Adriatic Sea	Surface	12.1 MPs/Kg (includes all kind of plastics)	Polyolefin > PE > PP > PET > PVC > PTU	Fragments > film > fibers
Abidli et al. (2018)	Southern Mediterranean	Surface	141 ± 25.98–461 ± 29.74 MPs/kg	PE > PP > PS	Fibres> fragments > foam > film > Pellets

Table 6.4. (Continued). Microplastics' studies at beaches in the Mediterranean Sea. In studies where many polymers were analysed, only the five most abundant are displayed.

	Location	Surface/Depth	Concentration	Polymer	Shape
Duncan et (2018)	North Cyprus	Both	45497 ± 11456 particles/m^3(average)	Not analysed	Fragments > foam > sheet-like > thread-like
Piperagkas et al. (2019)	Aegean Sea	Both	85 ± 41 MPs/kg	Not analysed	Fibres
Constant et al. (2019)	Gulf of Lyon	Surface	166 ± 205; 58 ± 53 MPs/kg	Fibres: polyester, acrylic and acrylamide Particles: PP, PE and PS	Fibres
This study (2020)	Western Mediterranean	Both	2.69 ± 1.23–19.24 ± 15.01 MPs/kg	PE > Rubbers > PP > EVOH > HDPE	Fibres

different areas and to take measures accordingly. Other factors, such as waste management, population awareness, and local policies, could also be influencing to great extent debris accumulation patterns.

Neither the season (beginning of summer or late autumn) nor the cleaning regime (automatic, manual or no cleaning) or the affluence of beach-goers (high, medium or low) seemed to cause significant differences between sampling locations. According to the number of visitors, we could expect to find less MPs in the protected La Punta beach, then in L'Alcatí and last in the urban beach (El Cabanyal); but this was not the case, probably due to the lack of cleaning at La Punta. Therefore, microplastics found in La Punta would mainly come from the sea, the wind or the weathering of bigger unremoved litter items (which are particularly abundant after storms). The same would apply to L'Alcatí, where we would expect also direct deposition by visitors, as in El Cabanyal. This relative homogeneity in our results may be consequence of several factors. First, the small size and low weight of microplastics make them easily spreadable by wind and sea currents. Another factor to consider is that these beaches are located below discharge waterways. These waterways carry irrigation water from the surrounding agriculture fields in L'Albufera coastal lagoon. This wetland is under great pressure from neighbouring villages, agricultural fields and industrial activities. Thus, the irrigation channels that are influenced by this lagoon end up carrying improperly disposed litter that comes from these sources. Eventually, all microplastics collected by these irrigation channels end up being discharged to the sea above L'Alcatí and La Punta beaches by the aforementioned mentioned waterways. The urban beach, El Cabanyal, faces a similar situation, as there is another waterway that discharges agricultural wastewater in its northern part of the beach. But also the port is in its southern part, acting as a barrier for dominant sea current and promoting the accumulation of sediments and, potentially, debris in this urban beach. As a consequence, these beaches could be receiving similar amounts of litter regardless of their degree of protection and their location. Grelaud and Ziveri (2020) obtained similar results when studying Mediterranean island beaches, although their monitoring strategy allowed to show that an increase of visitors actually yields an increase in the accumulation rate of beach litter. Although our results were not significant, microplastics at depth do seem sensibly higher in El Cabanyal beach, which is more exposed to visitors all the year round and is the venue of several mass events. A more intensive cleaning in this location could be helping to reduce microplastics input to the sediment and thus explain the non-significance of our results.

According to the CCI_{mp}, beaches were classified as dirty during the summer and clean to moderately dirty during the winter. However, CCI_{mp} only accounted for microplastics and therefore overlooked macrodebris in this study, even though it is known to be abundant in the beaches located at the natural park due to the different cleaning and beach management. Regarding the outliers detected, apparently, it is not rare to randomly find some extreme cases when sampling on sites where macrolitter has been degraded. Both outliers were found during the sampling in July, when the affluence of visitors is higher. These outliers may be consequence of one or several wrongly disposed bigger items that have been fragmented on site or that came from the same bigger parent litter item. Finally, mesoplastics and macroplastics were out of the scope of this study, but their presence was very noticeable on the surface during the samplings. Indeed, Grelaud and Ziveri (2020) showed that these categories were the most frequent types of debris on beaches, so definitely they have to be taken into account in future studies. In spite of the relatively low values, it is concerning finding microplastics of different types in every beach, and especially in a natural park. This shows that current measures of protection, public awareness and waste disposal are clearly insufficient.

Regarding the human activities in the area, it would be interesting to investigate waste input from some permanent sources, such as several WWTPs, agricultural lands and many commercial and recreational maritime routes close by. On the one hand, WWTPs have been recognized as contributors of microplastics to the sea (Roex et al., 2013) in (Bayo et al., 2020, Kazour et al., 2019). Characteristics of the microplastics found in WWTPS' effluents seem to depend not only on population habits but also on the activities carried out in the surroundings (Bayo et al., 2020). Agricultural lands and maritime activities are a recognized source of waste as well (Pérez et al., 2017, Uche-Soria and Rodríguez-Monroy, 2019, Bayo et al., 2019, Rochman, 2018, Argüello, 2020, Franco et al., 2020).

Microplastics' characteristics and polymers

There was a clear predominance of secondary microplastics over primary microplastics. This looks logical because the main activities in the area are recreational and agricultural. Industries that use preproduction pellets and similar items are not frequent in the area, although, interestingly, some white beads – like the ones found in this study – are commonly found in beaches at El Saler beaches, south of Valencia city. Indeed, this type of beads were also found in the digestive tracts of two striped dolphins stranded in the same area (Novillo et al., 2020). The origin of these primary microplastics is yet to be discovered and definitely needs to be addressed to stop their incorrect disposal or to fix possible leakages.

By far, the most frequent polymer found was PE. Other studies in the Mediterranean and in the world obtained this same result (Carson et al., 2011, Kaberi et al., 2013, Abidli et al., 2018, León et al., 2019). It is not surprising, as PE, including LDPE and HDPE, is the second most demanded resin in Europe for product fabrication and it is widely found in our everyday lives in a variety of items; e.g.: plastic bags, trays, toys and bottles (Plastics Europe, 2019).

Rubbers were the second most frequent group of polymers found. These elastomers are found in microplastics samples worldwide and usually come from the abrasion of tires on road surfaces (Halle et al., 2020). Latex was not included in the "rubber" category due to the natural origin of this elastomer, however, we included it in the analyses because it is not natural to find it in this ecosystem and it is debris as well. Indeed, it was the third most common polymer found right after rubbers (figure 6.4, b).

PP was the fourth most frequent polymer and it is the second most demanded resin in Europe (Plastics Europe, 2019). PP and EVOH (figure 6.4, b) are mainly used in food packaging; so it is probable that this has been the origin of many microplastics found in the present study, since many visitors choose these locations sampled to spend the day.

The least frequent polymers belonged to a mix of materials used in consumer products and industry, including some items that we could recognize as originated from paint, fishing lines and a piece of a car amber beacon. In any case, it is highlighted the urgency of setting up measures aimed to properly dispose and process these materials.

Implications for sea turtle nesting activity

Since 2001, a growing number of loggerhead sea turtle nests and nesting attempts have been recorded in the western Mediterranean coasts. In Spain this trend is notorious since 2014 (Carreras et al., 2018), and the beaches around Valencia are not an exception. In 2018, a loggerhead nesting female attempted to nest in the continuous beach north to El Cabanyal beach (Abalo-Morla et al., 2018).

Furthermore, due to its condition of full protection, La Punta beach is receiving eggs laid in different beaches of the Valencia region within a relocation program to protect the loggerhead turtle clutches. Among other anthropogenic threats, microplastics pose an added risk to these clutches. One hypothesis states that high concentrations of microplastics in sand can increase overall temperature due their higher specific heat (Wenn, 2007, Andrady, 2011, Beckwith and Fuentes, 2018), although specific heat varies among sediments. Hence, higher concentration of microplastics in the beach would lead to higher temperatures around the nests, consequently producing feminization of the clutches, and, in extreme cases, affecting the fitness or even the survival of the embryos (Nelms et al., 2016 and references therein). This potential increase of incubation temperatures due to microplastics in the substratum would worsen the alteration of sex ratio in many marine turtle nesting populations due to climate change (Nelms et al., 2016). Moreover, heat would potentially increase toxicants' leaching, including endocrine disruptors that could also contribute to biasing sex ratio (Yang et al., 2011). Eventually, all these factors would negatively affect reproductive success. However, there are more outcomes to consider. For instance, Carson et al. (2011) concluded the contrary: that microplastics accumulation in sand could decrease sediment's temperature, phenomenon that could cause the contrary effect: masculinization of the clutches. Furthermore, they also observed decreased permeability of the sediment, which could cause eggs dehydration (Carson et al., 2011). If this was the situation, reproductive success would be equally affected.

Local nest monitoring programs in La Punta beach show temperatures around the pivotal temperature, with similar production of both male and female hatchlings (Tomás, J., unpublished data). Thus, it is probable that no pollution stressors are affecting these nests yet. Although it is difficult to draw conclusions, nowadays it is improbable that microplastics accumulation in our area could affect nest temperature and permeability and thus sex ratio of the turtles' hatchlings. Similarly, Beckwith and Fuentes (2018) collected sand from the top layer at nesting beaches in Florida and found average amounts of 16.08 ± 34.61 pieces/m^2, far below the concerning concentrations found by Duncan et al. (2018) in Cyprus (45,497 ± 11,456 particles m^{-3}). Only (Duncan et al., 2018) sampled sand from the depth and obtained a mean of 5325 ± 3663 particles m^{-3} at 51–60 cm. Although we are starting to have data about microdebris in coastal environments, the lack of information about how this accumulation could be affecting granulometry, permeability and temperature of the sediments, does not allow to draw conclusions about how risky this type of pollution can be for marine turtles' development. Also, more research is necessary to quantify the amount of microdebris at depths where turtles lay their clutches. This information is important to identify increasing or decreasing trends and perform risk assessments.

Further steps

In spite of already having observational data, studies under controlled conditions are necessary to see whether microplastics in marine turtle's nests could be affecting embryonic development by leaching toxicants or modifying temperature and permeability around the nest environment, among other factors. Effect of neighbouring macroplastics in the incubation conditions of clutches must also be controlled.

Actions have to be taken also to identify the source and, hence, reduce the amount of litter reaching the environment. Microplastic waste is already part of our daily waste, so specific separation processes in WWTPs have to be considered. Also, national and local administrations, industries, services and citizens have to take part in a change of habits. Having healthy ecosystems is key to preserve habitats, biodiversity, cultural heritage, leisure, disturbance regulation, nutrient cycling and climate regulation (De Groot et al., 2002, Brenner et al., 2010, Loyoza et al., 2011, Loyoza et al., 2016).

Conclusion

Microplastics' concentration in our study area was among the lowest found among Mediterranean beaches. Therefore, the still sporadic nesting activity of loggerhead sea turtles does not seem to be threatened by these contaminants yet. Nonetheless, it is of concern that microplastics are also found in beaches under environmental protection figures and, according to CCI values, these beaches are classified as dirty or moderately dirty, thus showing continue monitoring microplastics because they are here to stay and if measure not implemented more items will reach the sea, potentially affecting the entire coastal and marine ecosystem. More research is essential to better understand the consequences that this emerging contaminant could have and how environmental conditions could determine the magnitude of this threat.

Supplementary material: table S6.1. Sampling sites where beach and samples were collected from the Valencia coast (Spain).

JULY 2018										
BEACH		**Cabanyal**			**BEACH**		**La Punta**			
Replicates		R1	R2	R3	**Replicates**		R1	R2	R3	
Coordinates	**Latitude**	39.4638	39.463605	39.463557	**Coordinates**	**Latitude**	39.31663	39.31589	39.315753	
	Longitude	-0.3214	-0.320049	-0.320069		**Longitude**	-0.2965	-0.296144	-0.296095	
NOVEMBER 2018										
BEACH		**Cabanyal**				**BEACH**	**La Punta**			
Replicates		R1	R2	R3	**Replicates**		R1	R2	R3	
Coordinates	**Latitude**	39.4638	39.4674	39.4658	**Coordinates**	**Latitude**	39.316278	39.315568	39.314817	
	Longitude	-0.3212	-0.3221	-0.3215		**Longitude**	-0.296088	-0.295901	-0.295509	

JULY 2018				
BEACH		**L'Alcatí**	**Natural Park**	
Replicates		R1	R2	R3
Coordinates	**Latitude**	39.321478	39.3219414	39.322297
	Longitude	-0.298717	-0.298912	-0.299212
NOVEMBER 2018				
Replicates		R1	R2	R3
Coordinates	**Latitude**	39.31648	39.317239	39.318048
	Longitude	-0.296351	-0.296654	-0.297

References

Abalo-Morla, S., Crespo J.L., Tomás, J., Merchán, M., Eymar, J., Marco, V., Belda, E.J., Revuelta, O., 2018. Exploring behavior of loggerhead turtle nesting females in the Spain's Mediterranean coasts through satellite tracking for clutch protection. *6th Mediterranean Conference on Marine Turtles*. Poreč (Croatia).

Abidli, S., Antunes, J. C., Ferreira, J. L., Lahbib, Y., Sobral, P., & El Menif, N. T. (2018). Microplastics in sediments from the littoral zone of the north Tunisian coast (Mediterranean Sea). *Estuarine, Coastal and Shelf Science*, 205, 1-9.

Alhammoud, B., Béranger, K., Mortier, L., Crépon, M., Dekeyser, I., 2005. Surface circulation of the Levantine Basin: comparison of model results with observations. *Progress in Oceanography* 66: 299-320. https://doi.org/10.1016/j.pocean.2004.07.015

Alkalay, R., Pasternak, G., Zask, A. 2007. Clean-coast index – A new approach for beach cleanliness assessment. *Ocean & Coastal Management* 50, 352 – 362. https://doi.org/10.1016/j.ocecoaman.2006.10.002

Alomar, C., Estarellas, F., Deudero, S., 2016. Microplastics in the Mediterranean Sea: deposition in coastal shallow sediments, spatial variation and preferential grain size. *Marine Environmental Research* 115: 1 – 10. https://doi.org/10.1016/j.marenvres.2016.01.005

Andrady, A. L., 2011. Microplastics in the marine environment. *Marine Pollution Bulletin* 62, 1596 - 1605. http://dx.doi.org/10.1016/j.marpolbul.2011.05.030.

Argüello, G., 2020. Environmentally sound management of ship wastes: challenges and opportunities for European ports. *Journal of Shipping and Trade* 5, 12. https://doi.org/10.1186/s41072-020-00068-w

Avio, C.G., Gorbi, S., Regoli, F., 2017. Plastics and microplastics in the oceans: From emerging pollutants to emerged threat. *Marine Environmental Research* 128, 2–11. https://doi.org/10.1016/j.marenvres.2016.05.012

Bayo, J., Rojo, D., Olmos, S., 2019. Abundance, morphology and chemical composition of microplastics in sand and sediments from a protected coastal area: The Mar Menor lagoon (SE Spain). *Environmental Pollution* 252, Part B, 1357 - 1366. https://doi.org/10.1016/j.envpol.2019.06.024

Bayo, J., Olmos, S., López-Castellanos, J., 2020. Microplastics in a urban wastewater treatment plant: the influence of physicochemical parameters and environmental factors. *Chemosphere* 238, 124593. https://doi.org/10.1016/j.chemosphere.2019.124593

Beckwith, V.K., Fuentes, M.M.P.B., 2018. Microplastic at nesting grounds used by the northern Gulf of Mexico loggerhead recovery unit. *Marine Pollution Bulletin* 131, 32–37. https://doi.org/10.1016/j.marpolbul.2018.04.001

Besley, A., Vijver, M.G., Behrens, P., Bosker, T., 2017. A standardized method for sampling and extraction methods for quantifying microplastics in beach sand. *Marine Pollution Bulletin*, 114(1), 77-83. https://doi.org/10.1016/j.marpolbul.2016.08.055

Booth, D.T., 2017. Influence of incubation temperature on sea turtle hatchling quality. *Integrative Zoology* 12: 352 - 360. https://doi.org/10.111/1749-4877.12255

Borrelle, S.B., Ringma, J., Law, K.L., Monnahan, C.C., Lebreton, L., McGivern, A., Murphy, E., Jambeck, J., Leonard, G.H., Hilleary, M.A., Eriksen, M., Possingham, H.P., De Frond, H., Gerber, L.R., Polidoro, B., Tahir, A., Bernard, M., Mallos, M., Barnes, M., Rochman, C.M., 2020. Predicted growth in plastic waste exceeds efforts to mitigate plastic pollution. *Science* 360(6510), 1515-1518. hhttps://doi.org/ 10.1126/science.aba3656

Bowman, D., Manor-Samsonov, N., and Golik, A., 1998. Dynamics of litter pollution on Israeli Mediterranean beaches: a budgetary, litter flux approach. *Journal of Coastal Research*, 14(2) 418 - 432.

Brenner, J., Jiménez, J.A., Sardá, R., Garola, A., 2010. An assessment of the non-market value of the ecosystem services provided by the Catalan coastal zone, Spain. *Ocean & Coastal Management* 53(1), 27 - 38. https://doi.org/10.1016/j.ocecoaman.2009.10.008

Carreras, C., Pascual, M., Tomás, J., Marco, A., Hochsheid, S., Castillo, J.J., Gozalbes, P., Parga, M.L., Piovano, S., Cardona, L. 2018. Sporadic nesting reveals long distance colonisation in the philopatric loggerhead sea turtle (*Caretta caretta*). *Scientific Reports* 8:1435 | doi:10.1038/s41598-018-19887-w

Carson, H.S., Colbert, S.L., Kaylor, M.J., McDermid K.J., 2011. Small plastic debris changes water movement and heat transfer through beach sediments. *Marine Pollution Bulletin* 62, 1708 - 1713. https://doi.org/10.1016/j.marpolbul.2011.05.032

Casale, P. and Tucker, A.D., 2017. Caretta caretta (amended version of 2015 assessment). *The IUCN Red List of Threatened Species 2017*. https://dx.doi.org/10.2305/IUCN.UK.2017-2.RLTS.T3897A119333622.en

Cole, M., Lindeque, P., Halsband, C., Galloway, T.S., 2011. Microplastics as contaminants in the marine environment: a review. *Marine Pollution Bulletin* 62(12): 2588 - 2597. https://doi.org/10.1016/j.marpolbul.2011.09.025

Constant, M., Kerhervé, P., Mino-Vercellio-Verollet, M., Dumontier, M., Vidal, A. S., Canals, M., & Heussner, S., 2019. Beached microplastics in the northwestern Mediterranean Sea. *Marine Pollution bulletin*, 142, 263-273.

De Groot, R.S., Wilson, M.A., Boumans, R.M., 2002. A typology for the classification, description and valuation of ecosystem functions, goods and services. *Ecological Economics* 41(3), 393 - 408. https://doi.org/10.1016/S0921-8009(02)00089-7

Duncan, E.M., Arrowsmith, J., Bain, C., Broderick, A.C., Lee, J., Metcalfe, K., Pikesley, S.K., Snape, R.T.E., van Sebille, E., Godley, B.J., 2018. The true depth of the Mediterranean plastic problem: Extreme microplastic pollution on marine turtle nesting beaches in Cyprus. *Marine Pollution Bulletin* 136, 334–340. https://doi.org/10.1016/j.marpolbul.2018.09.019

Fernandino, G., Elliff, C. I., Silva, I. R., Bittencourt, A. C. S. P., 2015. How many pellets are too many? The pellet pollution index as a tool to assess beach pollution by plastic resin pellets in Salvador, Bahia, Brazil. *Journal of Integrated Coastal Zone Management* 15(3), 325 - 332. https://doi.org/10.5894/rgci566.

Fisher, L.R., Godfrey, M.H., Owens, D.W., 2014. Incubation temperature effects on hatchling performance in the loggerhead sea turtle (*Caretta caretta*). *Plos One* 9 (12): e114880. https://doi.org/10.1371/journal.pone.0114880

Franco, A.A., Arellano, J.M., Albendín, G., Rodríguez-Barroso, R., Zahedi, S., Quiroga, J.M., Coello, M.D., 2020. Mapping microplastics in Cádiz (Spain): occurrence of microplastics in municipal and industrial wastewaters. *Journal of Water Process Engineering* 38, 101596. https://doi.org/10.1016/j.jwpe.2020.101596

Gago, J., Galgani, F., Maes, T., Thompson, R.C. 2016. Microplastics in seawater: recommendations from the Marine Strategy Framework Directive Implementation Process. *Frontiers in Marine Science* 3, 219. https://doi.org/10.3389/fmars.2016.00219

Galgani, F., Hanke, G., Werner, S., Oosterbaan, L., Nilsson, P., Fleet, D., et al., 2013. Guidance on monitoring of marine litter in European seas. In: EUR - *Scientific and Technical Research Series* - ISSN 1831 - 9424, eds G. Hanke, S. Werner, F. Galgani, J.M. Veiga, and M. Ferreira (Luxembourg: Publications Office of the European Union.

García-Ladona E. (2017) Currents in the Western Mediterranean Basin. In: Guillén J., Acosta J., Chiocci F., Palanques A. (eds) Atlas of Bedforms in the Western Mediterranean. *Springer*, Cham. https://doi.org/10.1007/978-3-319-33940-5_8

Godley, B.J., Broderick, A.C., Downie, J.R., Glen, F., Houghton, J.D., Kirkwood, I., Reece, S., Hays, G.C., 2001. Thermal conditions in nests of loggerhead turtles: further evidence suggesting female skewed sex ratios of hatchling production in the Mediterranean. *Journal of Experimental Marine Biology and Ecology* 263(1): 45 - 63. https://doi.org/10.1016/S022-0981(01)00269-6.

Gregory and Andrady, 2011. Microplastics in the marine environment. *Marine Pollution Bulletin* 62(8): 1596 - 1605. https://doi.org/10.1016/j.marpolbul.2011.05.030

Grelaud, M., Ziveri, P., 2020. The generation of marine litter in Mediterranean island beaches as an effect of tourism and its mitigation. *Science Reports* 10, 20326. https://doi.org/10.1038/s41598-020-77225-5

Gross, T.S., Crain, D.A., Bjorndal, K.A., Bolten, A.B., Carthy, R.C., 1995. Identification of sex in hatchling loggerhead turtles (*Caretta caretta*) by analysis of steroid concentrations in chorioallantoic/amniotic fluid. *General and Comparative Endocrinology* 99(2), 204 - 210. https://doi.org/10.1006/gcen.1995.1103

Halle, L.L., Palmqvist, A., Kampmann, K., Khan, F.R., 2020. Ecotoxicology of micronized tire rubber: past, present and future considerations. *Science of The Total Environment* 706: 135694. https://doi.org/10.1016/j.scitotenv.2019.135694

Hüffer, T., Metzelder, F., Sigmund, G., Slawek, S., Schmidt, T. C., Hofmann, T., 2019. Polyethylene micriplastics influence the transport of organic contaminants in soil. *Science of the Total Environment* 657, 242 - 247. https://doi.org/10.1016/j.scitotenv.2018.12.047

INE, Instituto Nacional de Estadística (España), 2021. Available at http://www.ine.es (Accessed 15th February 2021).

Jambeck, J. R., Geyer, R., Wilcox, C., Siegler, T. R., Perryman, M., Andrady, A., Narayan, R., Law, K. L., 2015. Plastic waste inputs from land into the ocean. *Science* 347 (6223), 768 - 771. https://doi.org/10.1126/science.1260352

Jung, M. R., Horgen, F. D., Orski, S. V., Rodriguez, V. C., Beers, K. L., Balazs, G. H., Jones, T. T., Work, T. M., Brignac, K. C., Royer, S., Hyrenbach, K. D., Jensen, B. A., Lynch, J. M., 2018. Validation of ATR FT-IR to identify polymers of plastic marine debris, including those ingested by marine organisms. *Marine Pollution Bulletin* 127, 704 - 716. https://doi.org/10.1016/j.marpolbul.2017.12.061

Kaberi, H., Tsangaris, C., Zeri., C., Mousdis, G., Papadopoulos, A., Streftaris, N., 2013. Microplastics along the shoreline of a Greek island (Kea isl. Aegean Sea): types and densities in relation to beach orientation, characteristics and proximity to sources. *Proceedings of the 4th International Conference on Environmental Management, Engineering, Planning and Economics (CEMEPE) and SECOTOX Conference*. ISBN: 978-960-6865-68-8, Mykonos Island (Greece).

Kaska, Y., Ilgaz, C., Özdemir, A., Baskale, E., Türkozan, O., Baran, I., Stachowitsch, M. 2006. Sex ratio estimations of loggerhead sea turtle hatchlings by histological examination and nest temperatures at Fethiye beach, Turkey. *Naturwissenchaften* 93: 338 - 343.https://doi.org/ 10.1007/s00114-006-0110-5

Kazour, M., Terki, S., Rabhi, K., Jemaa, S., Khalaf, G., Amara, R., 2019. Sources of microplastics in the marine environment: importance of wastewater treatment plant and coastal landfill. *Marine Pollution Bulletin* 146, 608 - 618. https://doi.org/10.1016/j.marpolbul.2019.06.066

Laglbauer, J. L. B., Franco-Santos, R. M., Andreu-Cazenave, M., Brunelli, L., Papadatou, M., Palatinus, A., Grego, M., Deprez, T., 2014. Macrodebris and microplastics from beaches in Slovenia. *Marine Pollution Bulletin* 89, 356 - 366. https://doi.org/10.1016/j.marpolbul.2014.09.036

Lebreton, L. C. M., Greer, S. D., Borrero, J. C., 2012. Numerical modeling of floating debris in the world's oceans. *Marine Pollution Bulletin* 64, 653–661. doi: 10.1016/j.marpolbul.2011.10.027

León, V. M., García, I., Moltó, V., González, E., Samper, R., Fernández-González, V., Muniategui-Lorenzo, S., 2018. Potential transfer of organic pollutants from littoral plastics debris to the marine environment. *Environmental Pollution* 236, 442 - 453. https://doi.org/10.1016/j.envpol.2018.01.114

León, V. M., García-Aguilera, I., Moltó, V., Fernández-González, V., Llorca-Pérez, L., Andrade, J. M., Muniategui-Lorenzo, S., Campillo, J. A., 2019. PAHs, pesticides, personal care products and plastic additives in plastic debris from Spanish Mediterranean beaches. *Science of The Total Environment* 670, 672 - 684. https://doi.org/10.1016/j.scitotenv.2019.03.216

Liubarsteva, S., Coppini, G., Lecci, R., Clementi, E., 2018. Tracking plastics in the Mediterranean: 2D Lagrangian model. *Marine Pollution Bulletin* 129: 151-162. https://doi.org/1016/j.marpolbul.2018.02.019

Lots, F. A., Behrens, P., Vijver, M. G., Horton, A. A., & Bosker, T., 2017. A large-scale investigation of microplastic contamination: abundance and characteristics of microplastics in European beach sediment. *Marine Pollution Bulletin*, 123(1-2), 219-226. https://doi.org/10.1016/j.marpolbul.2017.08.057

Loyoza, J.P., Sardá, R., Jiménez, J.A., 2011. A methodological framework for multi-hazard risk assessment in beaches. *Environmental Science & Policy* 14(6), 685 - 696. https://doi.org/10.1016/j.envsci.2011.05.002

Loyoza, J.P., Teixeira de Mello, F., Carrizo, D., Weinstein, F., Olivera, Y., Cedrés, F., Pereira, M., Fossati, M., 2016. Plastics and microplastics on recreational beaches in Punta del Este (Uruguay):

unseen critical residents? *Environmental Pollution* 218, 931 - 941. https://doi.org/10.1016/j.envpol.2016.08.041

Mansui, J., Molcard, A., Ourmières, Y., 2015. Modelling the transport and accumulation of floating marine debris in the Mediterraenan basin. *Marine Pollution Bulletin*, 91 (1): 249-257. https://doi.org/10.1016/j.marpolbul.2014.11.037

Mansui, J., Darmon, G., Ballerini, T., van Canneyt, O. van, Ourmières, Y., Miaud, C., 2020. Predicting marine litter accumulation patterns in the Mediterranean basin: spatio-temporal variability and comparison with empirical data. *Progress in Oceanography* 182: 102268. https://doi.org/10.1016/j.pocean.2020.102268

Martinez-Ribes, L., Basterretxea, G., Palmer, M., Tintoré, J., 2007. Origin and abundance of beach debris in the Balearic Islands. *Scientia Marina* 71, 305–314. https://doi.org/10.3989/scimar.2007.71n2305

Millot, C., 1987. Circulation in the western Mediterranean Sea. *Oceanologica Acta*, 10(2), 143-148.

Ministry of Agriculture and Fishing, Nourishment and Environment Technical Assistance in the Implementation of the Marine Strategy Frame Directive: Design, Development and Execution of the Monitoring Programmes., 2017. *Monitoring of Microparticles in Beaches (BM-6*), Spain.

Mrosovsky, N., Kamel, S., Rees, A.F., Margaritoulis, D., 2002. Pivotal temperature for loggerhead turtles (*Caretta caretta*) from Kyparissia Bay, Greece. *Canadian Journal of Zoology*, 80(12), 2118 - 2124. https://doi.org/10.1139/z02-204

Munari, C., Scoponi, M., & Mistri, M., 2017. Plastic debris in the Mediterranean Sea: Types, occurrence and distribution along Adriatic shorelines. *Waste Management*, 67, 385-391.

Murphy, F., Ewins, C., Carbonnier, F., & Quinn, B., 2016. Wastewater treatment works (WwTW) as a source of microplastics in the aquatic environment. *Environmental Science and Technology*, 50(11): 5800 - 5808. https://doi.org/0.1021/acs.est.5b05416.

Pasternak, G., Zviely, D., Ribic, C.A., Ariel, A., Spanier, E., 2017. Sources, composition and spatial distribution of marine debris along the Mediterranean coast of Israel. *Marine Pollution Bulletin*, 114(2): 1036 - 1045. https://doi.org/10.1016/j.marpolbul.2016.11.023

Pérez, I., González, M.M., Jiménez, J.L., 2017. Size matters? Evaluating the drivers of waste from ships at ports in Europe. *Transportation Research* Part D 57, 403 - 412. https://doi.org/10.1016/j.trd.2017.10.009

Piperagkas, O., Papageorgiou, N., & Karakassis, I., 2019. Qualitative and quantitative assessment of microplastics in three sandy Mediterranean beaches, including different methodological approaches. *Estuarine, Coastal and Shelf Science*, 219, 169-175.

Plastics Europe, 2019. Plastics - The Facts. Association of Plastic Manufacturers. http://ww.plasticseurope.org [Accessed May 2020]

Prevenios, M., Zeri, C., Tsangaris, C., Liubartseva, S., Fakiris, E., Papatheodorou, G., 2017. Beach litter dynamics on Mediterranean coasts: Distinguishing sources and pathways. *Marine Pollution Bulletin*. https://doi.org/10.1016/j.marpolbul.2017.10.013

R Core Team (2019). R: A language and environment for statistical computing. *R Foundation for Statistical Computing*, Vienna, Austria. URL https://www.R-project.org/.

Rochman, C.M., 2018. Microplastics research - from sink to source. *Science,* 360(6384), 28 - 29. https://doi.org/ 10.1126/science.aar7734

Roex, E., Vethaak, D., Leslie, H., Kreuk, M.D., 2013. Potential risks of microplastics in the fresh water environment. STOWA, Amersfoort. Technical report.

Sanjaume, E., Pardo-Pascual, J.E., 2011. Capítulo 10: Las dunas de la Devesa del Saler in: Las Dunas de España. Sanjaume Saumell, E. and Garcia Prieto, F. (Eds.), *Sociedad Española de Geomorfología*.

Schmidt., N., Thibault, D., Paluselli, A., Sémpére, R., 2018. Occurrence of microplastics in surface waters of the Gulf of Lion (NW Mediterranean Sea). *Progress in Oceanography*, 163, 214-220. https://doi.org/10.1016/j.pocean.2017.11.010

Simon-Sánchez, L., Grelaud, M., Garcia-Orellana, J., Ziveri, P. 2019. River deltas as hotspots for microplastic accumulation: the case study of the Ebro river. *Science of the Total Environment*, https://doi.org/10.1016/j.scitotenv.2019.06.168.

Standora, E.A., Spotila. J.R., 1985. Temperature Dependent Sex Determination in Sea Turtles. *Copeia* 3, 711 - 722. https://doi.org/10.2307/1444765

Suaria, G. and Aliani, S., 2014. Floating debris in the Mediterranean Sea. *Marine Pollution Bulletin*, 86(1-2): 494 - 504. https://doi.org/10.1016/j.marpolbul.2014.06.025

Uche-Soria, M., Rodríguez-Monroy, C., 2019. Solutions to marine pollution in Canary Islands' ports: alternatives and optimization of energy management. *Resources* 8(2), 59. https://doi.org/10.3390/resources8020059

Van Sebille, E., Wilcox, C., Lebreton, L., Maximenko, N., Hardesty, B. D., Van Franeker, J. A., et al.., 2015. A global inventory of small floating plastic debris. *Environmental Research Letters*, 10(12), 124006.

Villarrubia-Gómez, P., Cornell, S.E., Fabres, J., 2018. Marine plastic pollution as a planetary threat – The drifting piece in the sustainability puzzle. *Marine Policy*, 96, 213 – 220. https://doi.org/10.1016/j.marpol.2017.11.035

Vlachogianni, Th., Anastasopoulou, A., Fortibuoni, T., Ronchi, F., Zeri, Ch., 2017. Marine Litter Assessment in the Adriatic and Ionian Seas. IPA-Adriatic DeFishGear Project, MIO-ECSDE, HCMR and ISPRA. pp. 168 ISBN: 978-960-6793-25-7.

Wenn, J., 2007. Physical properties of polymers handbook. *Springer* New York, New York, 145 – 154. https://doi.org/10.1007/978-0-387-69002-5_9

Wickham, H., 2009. ggplot2: Elegant Graphics for Data Analysis. *Springer-Verlag* New York.

Wyneken, J. and Lolavar, A., 2015. Loggerhead sea turtle environmental sex determination: implications of moisture and temperature for climate change based predicions for species survival. *Journal of Experimental Zoology* 324B: 295-314. https://doi.org/ 10.1002/jez.b.22620

Yang, C. Z., Yaniger, S.I., Jordan, V.C., Klein, D.J., Bittner, G.D., 2011. Most plastic products release estrogenic chemicals: a potential health problem that can be solved. *Environmental Health Perspectives*, 119, 989–996. http://dx.doi.org/10.1289/ehp.1003220.

Zambiachi, E., Trani, M., Falco, P., 2017. Lagranian transport of marine litter in the Mediterranean Sea. *Frontiers in Environmental Science* 5:5. https:/ /doi.org/10.3389/fenvs.2017.00005

7. Evaluating the presence of microplastics in striped dolphins (*Stenella coeruleoalba*) stranded in the Western Mediterranean Sea.

Published in: Novillo, O., Raga, J. A., & Tomás, J. (2020). Evaluating the presence of microplastics in striped dolphins (*Stenella coeruleoalba*) stranded in the Western Mediterranean Sea. *Marine Pollution Bulletin*, 160, 111557. https://doi.org/10.1016/j.marpolbul.2020.111557

Abstract

Litter is a well-known problem for marine species; however, we still know little about the extent to which they're affected by microplastics. In this study, we analyse the presence of this type of debris in Western Mediterranean striped dolphins' intestinal contents over three decades. Results indicated that frequency was high, as 90.5% of dolphins contained microplastics. Of these microplastics, 73.6% were fibres, 23.87% were fragments and 2.53% were primary pellets. In spite of the high frequency of occurrence, microplastic amount per dolphin was relatively low and highly variable (mean ± SD = 14.9 ± 22.3; 95% CI: 9.58–23.4). Through FT-IR spectrometry, we found that polyacrylamide, typically found in synthetic clothes, was the most common plastic polymer. Here, we establish a starting point for further research on how microplastics affect this species' health and discuss the use of striped dolphins as indicators of microplastics at sea.

Introduction

Plastic is ubiquitous in the marine environment. Its input to seas and oceans has been increasing since the 1970s due to its availability and the ease and cost of production for both industrial and consumer use (Dauvergne, 2018). Jambeck et al. (2015) estimated that between 4.8 and 12.7 million tonnes of plastic were disposed in the oceans in 2010; and Eriksen et al. (2014) estimated that there were more than 5 trillion plastic pieces floating at sea, most of which seem to be microplastics (<5 mm, Gago et al., 2016; Galloway et al., 2017). On top of that, further increases in microplastic inputs are expected due to the massive consumption, misuse and mismanagement of personal protective equipment (PPE) and single-use plastic in the context of the COVID-19 pandemic (Fadare and Okoffo, 2020; Prata et al., 2020). Knowing if these microplastics are primary or secondary can provide us with information about the pollution source in different areas (Gago et al., 2016). For instance, primary microplastics are often manufactured in the form of microbeads, microfibres or glitter and are usually found in cosmetic products, textiles, air blasting and anti-fouling systems as well as in some drug delivery systems (Galloway et al., 2017; Guerranti et al., 2019; Tagg and Ivar do Sul, 2019). Alternatively, secondary microplastics are generated by the degradation of bigger plastic items that undergo physicochemical weathering when they are released into the environment. Degradation processes include photooxidation, mechanical breakdown and biodegradation (Gewert et al., 2015), and they can take up to years to completely break down plastic debris. For example, a bag made of polyethylene terephthalate (PET) can take more than 50 years to degrade completely under the sea in natural conditions (Webb et al., 2013). Furthermore, the rate at which we generate plastic is quicker than its degradation rate, so this contributes to further accumulation of plastics in the marine environment and, consequently, in marine biota.

Microplastics float and sink through the water column, where they can be ingested by marine organisms, either through direct capture or through feeding on previously exposed organisms. This way, microplastics potentially affect the entire food web (Choy et al., 2019; Nelms et al., 2019). Their presence and effects on small marine species, such as zooplankton, filter feeder invertebrates and some fish, is starting to be well documented (Cole et al., 2013; Karlsson et al., 2017; Messinetti et al., 2018). However, studies of microplastics in non-commercial large marine vertebrate species, such as cetaceans, are less common. Cetaceans are regarded as reliable sentinels of marine pollution due to their position at the top of the marine food web, conspicuous nature, and reliance on marine resources (Frias et al., 2010; Schwacke et al., 2013; Bakir et al., 2014; Ivar do Sul and Costa, 2014; Fossi et al., 2018). Up to date, 60% of cetacean species have been documented to have interacted with plastic items (Fossi et al., 2018) but only a handful of studies have looked for microplastics directly in their digestive tracts (table 7.1).

Marine debris, particularly macroplastics, affect cetaceans mainly by entanglement and/or ingestion, the later sometimes in massive amounts (e.g. Unger et al., 2016). When ingested,

Table 7.1. Published research articles reporting microplastics by directly looking at digestive tracts of cetaceans in Europe.

Species	**Location**	**Scientific articles**
Delphinus delphis	Ireland, United Kingdom and NW Spain	Curran et al. 2014; Hernández-González et al. 2018; Lusher et al. 2018; Nelms et al. 2019
Phocoena phocoena	Ireland and United Kingdom	Lusher et al. 2018; Nelms et al. 2019
Stenella coeruleoalba	Ireland and United Kingdom	Lusher et al. 2018; Nelms et al. 2019
Tursiops truncatus	Ireland and United Kingdom	Lusher et al. 2018; Nelms et al. 2019
Grampus griseus	United Kingdom	Nelms et al. 2019
Kogia breviceps	United Kingdom	Nelms et al. 2019
Lagenorhynchus acutus	United Kingdom	Nelms et al. 2019
Lagenorhynchus albirostris	Ireland	Lusher et al. 2018
Orcinus orca	Ireland	Lusher et al. 2018
Ziphius cavirostris	Ireland	Lusher et al. 2018
Megaptera novaengliae	The Netherlands	Besseling et al. 2015
Mesoplodon mirus	Ireland	Lusher et al. 2015

macroplastics may cause severe negative effects such as intestinal blockages or malnutrition, among others (Moore et al., 2013). Nevertheless, it is very unlikely that microplastics will cause severe damage or the death of these animals, as their size in relation to the organism's size is negligible. Notwithstanding, microplastics may hide other potential threats, such as their association with Persistent Organic Pollutants (POPs). Microplastics can adsorb high concentrations of POPs already present in the environment that eventually leak out into the organism's body; disrupting the function of their organs due to chronic exposure (Bakir et al., 2014). In addition, additives in their composition, such as phthalates, are suspected of endocrine disruption, and having a significant impact during the juvenile cetaceans' development as well as on the reproductive success in their adult stage (European Commission DG ENV, 2000). Due to all these reasons microplastic ingestion could represent an additional factor hindering cetacean populations' stability and survival, decreasing their fitness and adding to other stressors such as food depletion and infections (Fossi et al., 2018). Hence, large amounts of microplastics ingested over long periods of time may decrease the resilience and adaptability of cetacean populations. It is therefore of great importance to scan the occurrence, categories, and origin of microplastics in long-living marine vertebrates such as cetaceans (Schwacke et al., 2013; Fossi et al., 2018) in order to lay the foundations for future monitoring and experimental studies focus directly on risk, environmental and health assessments.

The present study arises from the lack of microplastic quantification in resident or transient cetaceans in the Mediterranean Sea, and from the need of comparing results from this region with other studies elsewhere. We study *Stenella coeruleoalba* (family Delphinidae), commonly known as the striped dolphin, because is the most abundant cetacean species along the western Mediterranean waters (Gómez de Segura et al., 2006; Aguilar and Gaspari, 2012). It inhabits temperate and subtropical waters of all oceans (Aguilar and Gaspari, 2012). In the Mediterranean, it is found mainly as resident species in open waters beyond the continental shelf (Notarbartolo di Sciara et al., 1993; Forcada et al., 1994; Gannier, 2005; in Aguilar and Gaspari, 2012), although more neritic incursions have been recorded during the last two decades (Aznar et al., 2017). The IUCN (International Union for the Conservation of Nature; Aguilar and Gaspari, 2012) classifies the Mediterranean subpopulation as vulnerable on the Red List of Threatened Species. Mediterranean Stenella coeruleoalba seemed to feed mainly on oceanic cephalopod species (Blanco et al., 1995; Meissner et al., 2012) but the diet may have shifted significantly in the last decades towards more neritic species such as juvenile hake (*Merluccius merluccius*) and southern shortfin squid (*Illex coindetii*) (Aznar et al., 2017).

Here we quantify and characterise by direct observation the microplastics in the digestive tract of a representative species from the Western Mediterranean Sea, the striped dolphin. We take advantage of the existence of a well-established stranding network in East Spain (see Material and Methods section), and the availability of fresh carcasses, to increase the knowledge about the interactions between microplastics and this pelagic – oceanic cetacean species.

Materials and methods

Study area

The Valencian Community is located in the East of Spain (from 40°31' N – 0°31' E to 37° 51'N – 0° 45'W, figure 7.1). Its coast is 518 km long and it is divided into three provinces: Castellón (139 km long), Valencia (135 km long), and Alicante (244 km long), north to south. In total, it has 4,935,010 inhabitants. Including their metropolitan areas, Valencia is the most populated area (1,550,885 inhabitants), followed by Alicante (452,462 inhabitants) and Castellón (144,446 inhabitants, INE, 2019). All three cities are located on the coast. Tourism is the most important economic activity of the region, especially during the summer months. In 2019, 27.8 million tourists visited the Valencian Community (Turisme Comunitat Valenciana, 2019).

The main coastal surface sea current flows from north to south and several rivers outflow in the area. The most important one is the Ebro River, which outflows just above the northern limit of the Valencian Community (figure 7.1).

Gut content analysis

Digestive tracts (N = 43) were obtained from necropsies of fresh striped dolphins' carcasses. Strandings and necropsies in the region are managed by a coordinated network formed by private and public institutions (Tomás et al., 2008). All dolphins were found stranded dead on beaches from the Valencian Community between 1988 and 2017, with the exception of

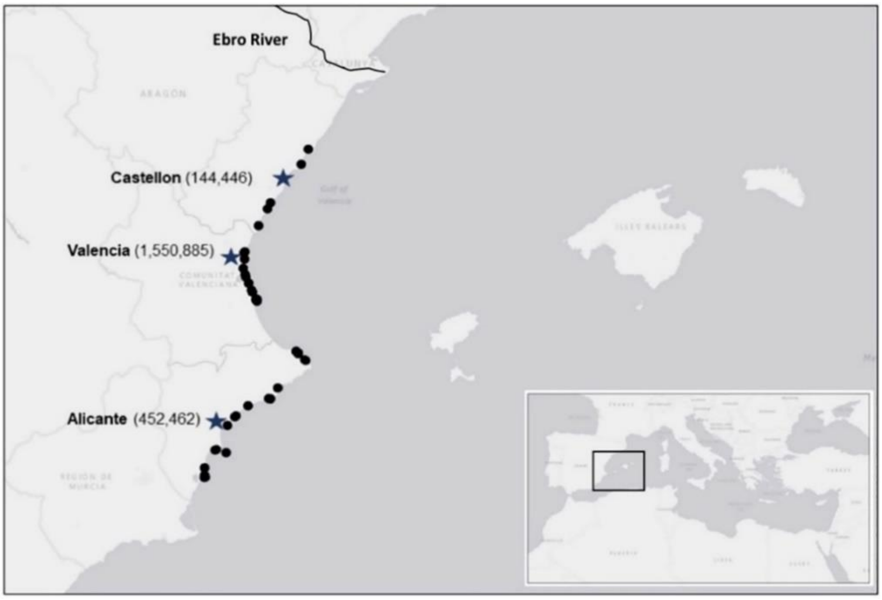


Figure 7.1. Map of the Valencian Community. Black dots indicate the stranding locations of the striped dolphins analysed in the present study. Names of the three most populated cities and urban areas are written in bold and marked with a star followed by their number of inhabitants in brackets. Source: ArcMap.

one that died at the rescue center. All the animals used for analyses were in apparent good condition according to body shape, except one that was reported as thin during the examination previous to the necropsy. We classified the state of the carcasses by following the criteria proposed by Geraci and Lounsbury's (2005) stranding field guide. In this scale, 1 means "alive"; 2, "freshly dead"; 3, "starting decomposition but organs basically intact"; 4, "advanced decomposition"; and 5, "mummified or skeletal remains only". Only individuals belonging to states 2 (75.6%) or 3 (24.4%) were taken into account for the analyses. Choosing states 2 and 3 guarantees that the carcass was not floating in the sea or laying in the sand for a long time before the examination and necropsy; reducing potential post-mortem contamination. Furthermore, none of the dolphins presented a meaningful amount of sand in mouth or upper digestive tract; which further confirms that sand-borne contamination was residual. We used sterile and stainless steel material in all the necropsies. Digestive tracts prior to 2017 (N = 30) were closed with natural-fibre strands and frozen immediately at −20 °C after necropsy and thawed at room temperature for the study; while digestive tracts from 2017 (N = 11) were dissected during the necropsies.

We analysed digestive contents for microplastic detection following Lusher et al. (2015) and Foekema et al. (2013) protocols with few adaptations, but always took care to avoid contamination. We washed the contents through 200 µm and 100 µm nested sieves. Prior to microplastic analysis, parasites and diet items were rinsed over the sieve and then separated for further studies. After this step, contents were introduced into glass bottles with a filtered 10% KOH solution, 3 times the volume of the contents during three weeks. After digestion, we filtered the resulting solutions under vacuum using a Büchner filter and GF/C microfiber filters in a type I laminar flow cabinet with positive pressure to guarantee that external air-borne particles could not contaminate de samples. When remains were too complex and dense to accurately separate microplastics, we resuspended the digestion product with a supersaturated solution of NaCl (140 g/L) and let it set for 24 h. Afterwards, the supernatant was re-filtered under vacuum as mentioned above. We identified the particles retained on the filters with a dissecting microscope (Leica MZ APO, 8–80×). When doubts arose concerning the nature of some particles, each particle was exposed to a hot needle to see whether it changed its shape, hence confirming its plastic composition (Hanke et al., 2013).

We classified plastic items into categories according to their shape and colour, following the protocol used by the Harbour and Coasts Study Centre (Spain, Ministry of Agriculture and Fishing, Nourishment and Environment, 2017). Only microplastics, i.e. plastic items smaller than 5 mm (GESAMP, 2015; Gago et al., 2016), were considered for the analyses.

Polymer analyses

After the classification and quantification of items, we kept 30 microplastic items for polymer identification. These items were selected randomly. We identified polymers by ATR FT-IR (Attenuated Total Reflection Fourier Transformed Infrared Spectroscopy) with an Agilent Technology Cary 630 spectrometer. We thoroughly cleaned the ATR diamond and its base with ethanol before and after procedure, between every sample and in between measurements of the same sample. Before every sample scan, the spectrometer scanned the background 8 times. Microplastics are solid samples that do not need to be included in any particular material; hence, the background was the air filling the working space. The experimental nominal working resolution was 4 and the apodization function used was Happ-Genzel. We analysed each sample three times in order to assure accuracy; and in each measurement, 8 scans were performed. We used ATR as the measurement mode and set the wavelength range to 4000–650 cm^{-1}. We compared the resulting spectra with those from our custom polymer library and only accepted matches above 70% as valid. All samples scoring under 70% were classified as "Unidentified". We analysed all the spectra using the spectrometer's native software Agilent Microlab.

Contamination control

Blank samples (GF/C filters) were exposed to open air in the laboratory at the same time digestive contents were filtered through nested sieves in the necropsies' facilities in order to assure that air-borne contamination was produced. Procedural blanks were subjected to the same process as the digestive tract samples. Tap water was tested for the presence of microplastics by previously filtering 5 L following the same procedure than with the samples and the procedural blanks. KOH solutions were prepared with Milli-Q water and filtered before use. Only glass and stainless steel material was used to manipulate and store the samples. White cotton laboratory coats and blue nitrile gloves were used during the whole process, and single-use gowns were used on top of them during necropsies according to health and safety protocols. Sponges used for surface cleaning were always made of the same material and colour (bright yellow) so as to identify potential contamination quickly. Any plastic item found in samples with the same characteristics of the tools used in the working space was discarded to prevent false positives. All materials were thoroughly cleaned with filtered deionized water, soap and ethanol 70%; and then checked under a dissecting microscope for any microplastic presence prior to analysis.

Statistical analyses

Microplastic content information and biometric data were registered in a Microsoft Excel (version 16.4.1) spreadsheet. We calculated 95% confidence intervals for the mean microplastic content, excluding cases without microplastics, by bootstrapping 10,000 replications in the free software QPweb v1.0.13. Likewise, the confidence interval for prevalence was obtained by Sterne's method. We calculated the rest of the analyses using R Studio version 1.0.143. Specifically, we explored data with descriptive statistics and checked the normality of these data using both graphical tools and the Shapiro-Wilk test. To explore temporal changes, we separated microplastic content per individual into two groups of dolphins collected in two separated periods, 1989–2007 and 2010–2017, since there were no dolphins collected in the period 2008–2009. We used the Mann-Whitney-U test to check for differences between these groups. Finally, we used the Kruskal-Wallis test to see whether location, sex and state of the carcass had any effects on the amount of microplastics ingested by dolphins.

Results

Dolphin biometrics and microplastic counts

Mean total length (±SD) of dolphins was 160.31 ± 66.38 cm. According to their body length, they were all adults or late juveniles. Also, 55.8% were males (N = 24) and 44.2% females (N = 19), Location, biometric data and other information are provided in the Supplementary material.

Of the analysed dolphins, 90.5% contained microplastics. We collected a total of 672 items, of which 73.6% were fibres, 23.87% were fragments and 2.53% were industrial pellets. Mean number of microplastic items per striped dolphins was 14.9 ± 22.3 (mean ± SD; 95% CI: 9.58–23.4). Microplastic amount varied highly among individuals, so data did not follow a normal distribution (as proved by the Saphiro-Wilk test and graphic analyses). Therefore, calculating the median seemed more representative of the microplastic amount per dolphin (median = 5, range = 0–82). When considering only dolphins with microplastics, median increased to 7 items per individual (range: 1–82). The striped dolphin with the highest number of microplastic items was found stranded in Orihuela (Alicante province) in February 2017. This dolphin had 45 translucent fragments, 29 black fibres and 8 red fibres. Following this individual, another striped dolphin, stranded in the municipality of Javea (Alicante province), contained 79 microplastics; these items being 45 red fibres and 34 black fibres. The next highest one was stranded in Cullera (Valencia province) in 2015, and contained 74 microplastic items of which 17 were white spherical pellets (primary microplastics). Apart from the pellets, it contained 22 black fibres, 21 translucent fibres, 12 red fibres and 1 green fibre. Only one other individual stranded in Alboraya in 2016 (Valencia province) had primary microplastics, these ones being very similar to the ones found in the dolphin from Cullera. Although these two dolphins stranded in different years and were not related, it is remarkable that the same kind of pellets were found in two relatively close locations around the urban area of Valencia City, 40 km apart from each other.

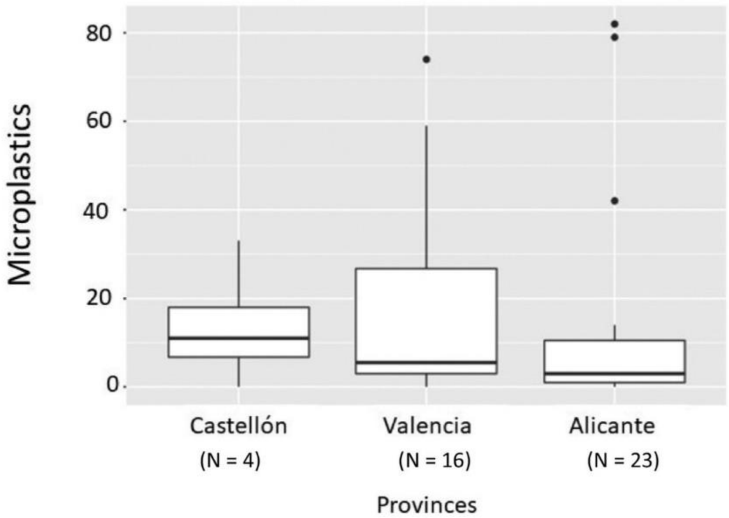


Figure 7.2. Number of microplastics found in striped dolphins per province of the Valencian Community north to south. In brackets, number of dolphins analysed per province.

Overall, microplastic content in striped dolphins was relatively low. In most cases, particles found were below 20 per individual and, as said before, only 3 of them had more than 70 microplastic items. We can see in figure 7.2 that variability in microplastic content was highest in the province of Valencia and smallest in the province of Alicante; although differences in microplastic amount per dolphin between provinces were not significant (Kruskal-Wallis test, $p > 0.005$).

Microplastic colours and polymers

In total, the predominant microplastic colour found in the dolphins was black (50.1%), followed by red (21.2%), translucent (10.9%), white (3.8%), and other less frequent colours, such as yellow (figure 7.3). Specifically, most of the fibres were black (68.4%), followed by red (23.2%), translucent (5.1%), green (2.9%), white and yellow (0.2% each, see figure 7.3). On the other hand, colour of fragments was mostly translucent (46.8%), followed by white (15.8%), black (15.2%), red (2.5%), green and others (1.3% each, figure 7.3). Table 7.2 shows total mean and median items per colour, together with their confidence interval.

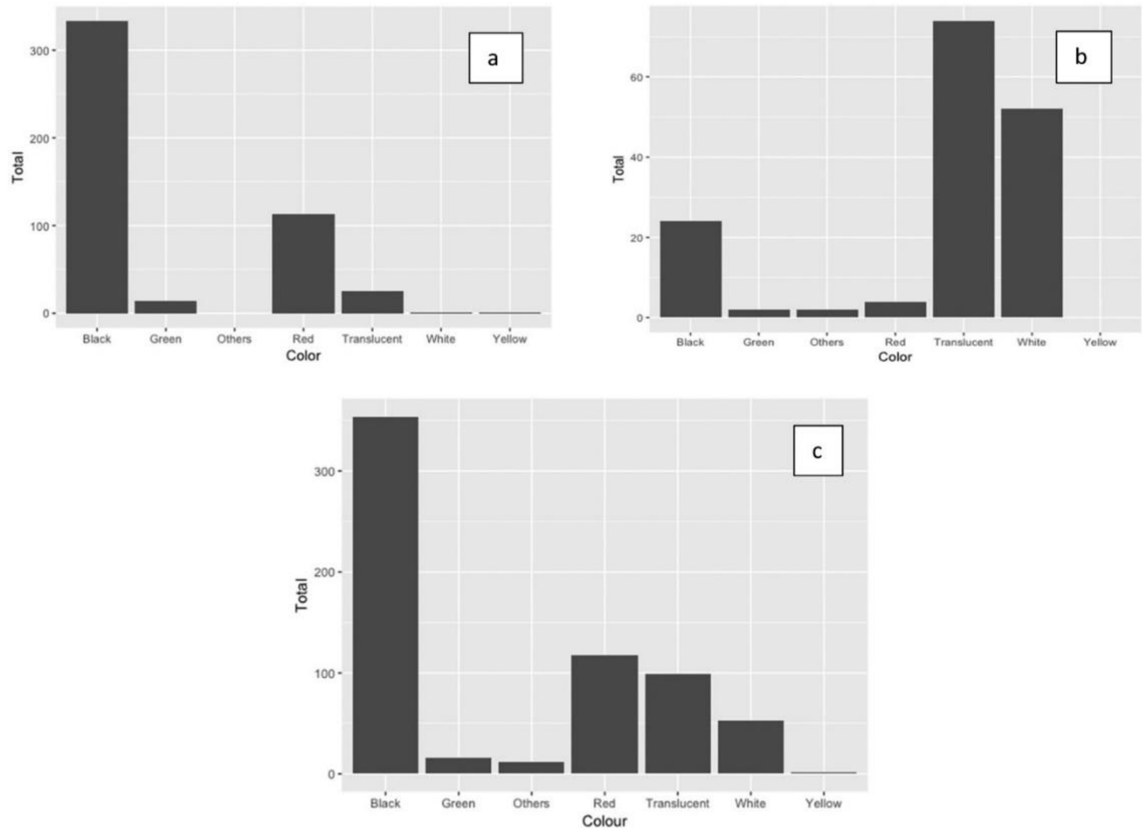


Figure 7.3. Frequency of occurrence of microplastic colours found in striped dolphins from the Valencian Community waters. (a) Colour of fibres, (b) colour of fragments, (c) total colours found in both fibres and fragments.

From items analysed by FT-IR, 40.9% were of polyacrylamide, 27.3% were PET (polyethylene terephthalate), 13.6% were alginic acid and 9.1% were HDPE (high density polyethylene) (figures. 7.4, 5, table 7.3). Of these polymers, 73% were synthetic polymers, 15.4% were not plastic polymers (alginic acid and 1 sample identified as cellulose filter paper) and 11.5% were unmatched (under 70% of match). Polymers identified as non-plastics were excluded from the analyses. Concerning the cellulose filter paper, it could not be considered as contamination during the laboratory procedure since we did not use cellulose filter papers at any time. Notably, all of the items identified as PET were specifically linked as belonging to polyester fibres (see table 7.3).

Table 7.2. Microplastics prevalence by colour in striped dolphins from the Valencian Community waters. Mean ± SD, median, range, and confidence intervals were calculated excluding individuals with no microplastics (N = 38). In "Others", CI (95%) for the mean could not be calculated due to the constant nature of the variability.

	Black	**Red**	**White**	**Translucent**	**Green**	**Others**
Prevalence (%)	76.7	46.5	11.9	30.2	20.9	6.9
Mean ± SD	10.72 ± 11.53	5.85 ± 9.79	11 ± 5.79	7.62 ± 12,34	1.78 ± 0.83	1 ± 0
Median	5	3	11	3	2	1
Range	1–37	1–45	2–17	1–45	1–3	1
CI (95%) for the mean	7.15–15.2	3.25–14.3	5–14.4	3.15–18.7	1.22–2.22	–
CI (95%) for prevalence	61,7–87.6	32.5–61.7	4.8–52.9	18.4–45.3	11.1–35.9	0–0.12

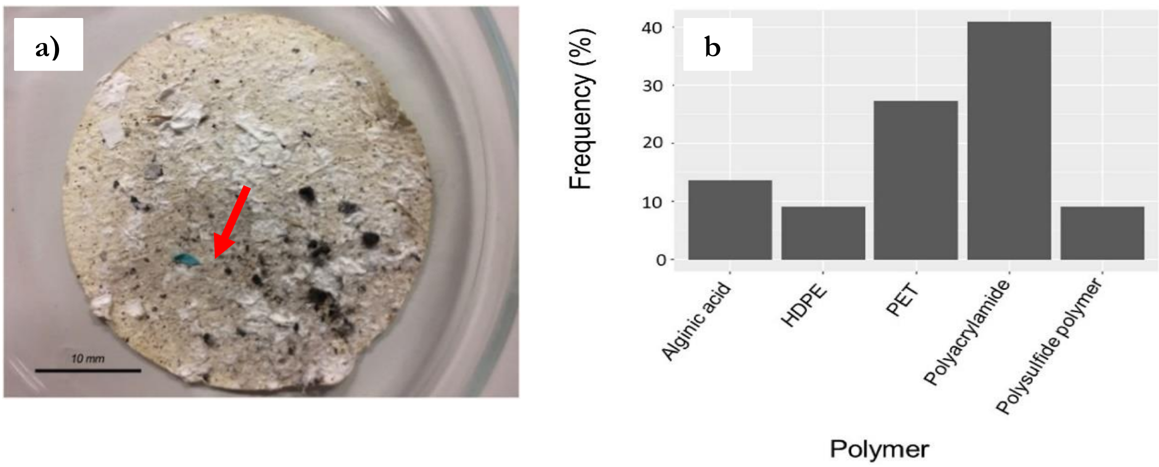


Figure 7.4. a) Debris resulting from KOH (10%) digestion of gut contents of one striped dolphin stranded in the Valencian Community coast on a GF/C microfiber filter. Red arrow indicates the presence of a blue microplastic; b) frequency of polymers found by FT-IR analysis (n = 30) in striped dolphins stranded in the Valencian Community coasts. HDPE: high density polyethylene, PET: polyethylene terephthalate (polyester).

Table 7.3. Type of microplastic analysed by FT-IR and reliability of the match with the corresponding polymer. Microplastics with a reliability lower than 0.7 were discarded from the analysis. PET: polyethylene terephthalate, HDPE: high density polyethylene.

Type	**Polymer**	**Reliability**
Fibre	Polyacrylamide	0,8509
Fibre	PET	0,7909
Fibre	Polysulfide polymer	0,7347
Fibre	Polysulfide polymer	0,7347
Fibre	Polyacrylamide	0,85609
Fibre	PET	0,79609
Fibre	PET	0,79609
Fibre	Polyacrylamide	0,85609
Fibre	PET	0,79609
Fibre	PET	0,79609
Fibre	PET	0,79609
Fibre	HDPE	0,82155
Fibre	Polyacrylamide	0,7367
Fibre	Latex	0,600
Fibre	Alginic acid	0,86094
Fibre	Polyacrylamide	0,78268
Fragment	Alginic acid	0,79238

Table 7.3. (Continued). Type of microplastic analysed by FT-IR and reliability of the match with the corresponding polymer. Microplastics with a reliability lower than 0.7 were discarded from the analysis. PET: polyethylene terephthalate, HDPE: high density polyethylene.

Type	Polymer	Reliability
Fragment	Polyacrylamide	0,83886
Fragment	Natural latex	0,6
Fibre	Alginic acid	0,8508
Fibre	Polyacrylamide	0,77633
Fibre	Polyacrylamide	0,77633
Fragment	HDPE	0,76882
Fibre	Polyacrylamide	0,86652
Fragment	Cellulose filter paper	0,82174
Fragment	Natural latex	0.6
Fragment	HDPE	0,82155
Fibre	Unidentified	Fail
Fibre	Unidentified	Fail
Fibre	Unidentified	Fail

Temporal and sex effects on microplastic amount

Concerning changes over time, no significant differences in microplastic contents, colours or shape were found between dolphins collected during the period 1989–2007 and dolphins collected during the period 2010–2017, nor between males and females or state or carcass (U-Mann-Whitney, $p > 0.005$; Kruskal – Wallis, $p > 0.005$, respectively).

Blanks and contamination control

Procedural and air-borne contamination control blanks were all clean. Nevertheless, two items were discarded because they were very similar to working materials (nitrile gloves and yellow sponge). Finally, three microplastics were found from the filtered tap water, so we subtracted them from the total counts.

Discussion

Microplastic contents

The number of microplastics found per dolphin was relatively low for their body size. Approximately 50% of the individuals had less than 5 items, and if we look closely, 25% of the dolphins had just one microplastic item or no microplastics at all. Dolphins with high microplastic abundance were not frequent and presented secondary microplastics made of the same colour and consistency. An explanation for this could be that all of those items were generated from a single and bigger piece of plastic, although we cannot prove nor assure this statement. Although low, average microplastic content is similar to the amounts found in other cetacean species (table 7.4) and higher than in fish species from the area. For instance, Nadal et al. (2016) found an average of 3.75 microplastics per individual in *Boops boops*, Alomar and Deudero (2017) found 0.34 microplastics per individual in *Galeus melastomus* and Compa et al. (2018) found 0.21 microplastics per individual in *Sardina pilchardus* and 0.18 ± 0.20 in *Engraulis encrasicolus*. Regarding microplastics in Mediterranean waters, de Haan et al. (2019) found 0.18 ± 0.16 items/m2 on average at the sea surface. Due to these low numbers, and also to the small size of microplastics compared to dolphin size and digestive tract length, these results have to be taken with caution when considering potential impacts on dolphins' health. It is not likely that microplastics themselves pose a physical threat to this species. However, a chemical threat cannot be ignored. Substances present on plastics, such as POPs and endocrine disruptors, are of concern. They can be either part of or adsorbed to microplastics and contribute to the total burden of chemicals to which animals are exposed at sea (Gallo et al., 2018). Endocrine disruptors can be a potential threat for population stability, causing low reproduction rate and hormonal dysfunction (Gallo et al., 2018).

In the present study fibres were more abundant than particles, and both categories were more abundant than primary microplastics. Primary microplastics were found just in two dolphins and all were white industrial pellets. Neither glitter particles nor cosmetic microbeads were found. This is in line with the results of other studies performed in marine mammals and top predator fishes in the European waters of the Atlantic Ocean (Lusher et al., 2015; Bellas et al., 2016; Lusher et al., 2018; Nelms et al., 2018; Nelms et al., 2019), as well as with the results obtained in analyses of waters and sediments of the Ebro river (East Spain, Simon-Sánchez et al. 2019). In fact, after extensive literature research, we found that prevalence of pellets and primary microplastics seems to be low in all studies. This may indicate that sources of industrial pollution are less important than pollution by citizens that

Table 7.4. Microplastics found throughout cetacean species in the later years.

Study	Microplastics	Fibres (%)	Fragments (%)	Polymer analysis (RAMAN or FTIR)
Present study	14.9 average/individual	73.6	23.87	Yes
Nelms et al., 2019	5.5 average/individual	84	16	Yes
Hernández-González et al., 2018	12 average individual	96.59	3.16	No
Lusher et al., 2018	1–88 (range)	83.6	16.4	Yes
Lusher et al., 2015	29 mps in one individual	58	42	Yes
Besseling et al., 2015	16 items in one individual (mps amount not specified)	25	75	Yes
Curran et al., 2014	1 in one individual	Not specified	Not specified	No

consume plastics, or that they are less accessible to marine fauna, not entering in the food webs as other types of microplastics.

As for microplastic colours, the most abundant and frequent was black, followed by red, translucent, white and others (table 7.2). Other studies performed in the Atlantic Ocean and other areas of the Mediterranean Sea got similar results to ours (Bellas et al., 2016; Nelms et al., 2018), with dominance of black, red, translucent and white; although the most extensive study performed up to date (Lusher et al., 2018) reports black in the third place, with its ranking being headed by blue and grey. Unlike in the present study, none of these previous studies distinguished in between the colour of fibres and particles. We believe that this is something to consider in such type of studies, since colours of the plastics found may help in identifying their origin and to study whether there is a link between the colour and the different type of polymers. However, this approach has limitations because colour is eventually washed out due to weathering in the environment and also during the digestion process in the digestive tracts.

In 2015, Spain was the destination with the highest number of foreign tourists in Europe, and it is always among the top four destinations receiving the most tourists in the continent (Eurostat, 2017). Valencia is the most populated province of the Valencia Community, although all three provinces multiply their population during spring and summer months, as mentioned above. These visitors add to the basal population, potentially increasing debris input to the sea, either directly at the beaches or through consumption elsewhere. Moreover, the Ebro River, which is the river carrying the largest water flow in the Spanish Mediterranean, outflows just above the northern limit of the Valencian Community (figure 7.1). Several studies have pointed out that this river carries important amounts of microplastics in its waters, being most of them fibres (Simon-Sánchez et al., 2019 and references therein). Ebro River influence on Valencian Community waters is very important due to the dominant coastal current that flows from north to south along the coast (André et al., 2005), potentially carrying contaminants and debris discharged by the Ebro River along with it. According to the model designed by Liubartseva et al. (2018), Catalonian waters (northeast Spain) are the second biggest hotspot for microplastic accumulation in the whole Mediterranean Sea, just headed up by the Sicilian sub-basin and followed by the Po River Delta in Italy. Additionally, the coast of the Valencia province (figure 7.1) also appears as one of the areas having the highest densities of plastic debris at the sea surface in the Mediterranean Sea (Liubartseva et al., 2018). In fact, plastic debris have been reported as highly frequent in marine species from the same area, such as the loggerhead sea turtle (Domènech et al., 2019 and references therein). Knowing riverine input dynamics into the sea and sea currents' flow direction is essential in order to identify pollution sources and the movement of contaminants. Nevertheless, there are not many studies available in this aspect and there are a lot of knowledge gaps (Guerranti et al., 2020).

The most abundant polymer in the analysed dolphins was polyacrylamide, a water-soluble polymer. It is used as a thickening and absorbing agent, serving applications in soil conditioning, cosmetics, flocculation operations, and oil recovery tasks. It can polymerize in the presence of free radicals (European Chemicals Agency, 2017). It may be found at sea due to the high amount of cosmetics used by population. Or it could appear as a subproduct of oil extractions, such as the one existing just above the northern limit of the Valencian Community, in the surrounding waters of the Ebro River delta. Nowadays, there is the active Casablanca oil platform in Tarragona waters (south Catalonia), which has had at least one known spill of 8000 L of crude oil in the area, (De la Torre and Albaigés, 2016; BOE 301, 2018). However, in our study, items containing polyacrylamide (all of them fibres, see table 7.3) probably came from clothes and other textiles. Acrylic polymeric structures are very difficult to recycle because of their structure, unlike other plastics classified as thermosets (Association of Plastics Manufacturers, 2018). Few studies also mention the finding of polyacrylamide and acrylic polymers in cetaceans (Lusher et al., 2015; Neves et al., 2015; Nelms et al., 2018) but its origin is not discussed. Concerning other materials found, polysulfide indicates the presence of rubber. Rubber can come from several consumer goods, such as shoes and car wheels. HDPE (high density polyethylene) is used in plastic bottles, plastic bags, food containers and a wide range of consumer products. It can be recycled, although large quantities of these materials are not properly disposed of and, therefore, end up in the ocean. We also detected alginic acid in two samples. Alginic acid is a polysaccharide found in algae cell walls and biofilms that resists KOH digestion. Because of its organic origin, we subtracted these items from the count. Some samples contained algae, thus, it could be possible that some plastic samples were covered by this substance. In order to avoid these identification problems, we would suggest a thorough rinsing with deionized water, as suggested by Jung (2018).

Polymer composition in our analysis differs from another study using FT-IR on samples from a true beaked whale (*Mesoplodon mirus*) in Ireland (Lusher et al., 2015). These authors analysed 80 microplastic items that corresponded to, in descending order of abundance: rayon, polyester, acrylic and polypropylene. However, we can see some similarities with our results, since in our study, acrylic polymers (specifically, polyacrylamide) and polyester were also abundant. Even more different results were found in the study of Nelms et al. (2018) who analysed grey seal scats (*Halichoerus grypus*), and found that PET constituted only 28% of the samples, while PP and ethylene propylene were more abundant. In another study on fish species in Portugal (Neves et al., 2015), PP was also the most abundant polymer found, followed by PET, resin, rayon, polyester, nylon and acrylic. Sample sizes in all of these studies, as in ours, are too small to draw conclusions about differences in polymer composition among species and locations. Analysing 10% of the items found is important to get a minimum reliability. This was a limitation in our study, but we highly encourage the standardization of this protocol to determine the origin of the microplastics found and to carefully watch out for items that result to not be plastics.

Analysing the amount and composition of plastics in striped dolphins helps us to understand the extent to which microplastics are present in marine organisms and to assess whether they pose a risk to the dolphin population or not. Furthermore, they are at the top of the trophic chain, which can give us an idea about the accumulation of plastics in predators and in higher trophic levels than other organisms normally used, such as filter feeders. Together with data about other species, we would be able to perform detailed studies about microplastics' distribution in the trophic chain. However, the use of striped dolphins as bioindicators of environmental health is not ideal. Firstly, this is a protected species and getting the carcasses depends on the presence of a well-established stranding network, and fresh carcasses are not easy to obtain. Secondly, processing their long digestive system is very time consuming and it is sometimes difficult to control air-borne and tap water contamination. Moreover, most of the rescue centres that also gather data do not have the proper facilities to do so. And finally, the digestion process can make the separation process difficult in dolphins that have recently eaten; and microplastics weathering can make polymer identification a real challenge. We think that it is of interest to monitor microplastics in cetaceans, but smaller species such as fish and molluscs seem more appropriate for routine checks on marine pollution, environmental status and water quality due to their size and ease of sampling and processing. Nonetheless, below we further discuss the role of striped dolphins as indicators of microplastics at sea.

According to studies performed by Plastics Europe (2018), recycling of plastics has increased 79% in a 10-year period, plastics used as energy recovery have increased 61% and landfill disposal of plastics has decreased 43% (2006–2016) in Europe. However, despite these figures, the proportion of recycled plastics worldwide is still small, and Spain is trailing behind countries from northern and central Europe. On top of that, the harm to the environment caused by synthetic fibres, the most common type of microplastic found in the present study, has yet to be completely understood and, therefore complicates the search for alternatives that would allow dealing with this specific item. Long-term monitoring is necessary in order to see temporal trends of microplastic presence in biota and create appropriate models that would allow describing debris distribution and, therefore, propose appropriate mitigation plans.

Microplastic analyses' advantages and limitations

Data collected through opportunistic sampling by stranding networks can be biased due to seasonal and spatial variation in natural factors and collection effort. Nevertheless, having constant protocols over years and discarding animals in bad condition gives us some consistency in the observations (Hart et al., 2006; Witt and Godley, 2007; Tomás et al., 2008). As a result of this, we were able to analyse a relatively large sample size of this dolphin species (N = 43), which is unusual when studying a wild protected species. In opposition to our study, studies on detection of plastic in cetaceans in the Mediterranean have focused on biomarker studies so far (Fossi et al., 2012, Fossi et al., 2014, Fossi et al., 2016). However, biomarker methods, such as testing the presence of phthalates in tissues, cannot assure whether the origin of these phthalates are microplastics, as they can be leaked from different materials like waterproof membranes or macroplastics. Likewise, biomarker studies using the oxidative response of organisms cannot fully link biochemical stress to specific factors such as interaction with plastics, given that these responses are general defensive reactions and can be triggered by a variety of reasons, including stress caused by predators (Lusher et al., 2018). Taking into consideration the advantages and limitations that each approach has, we do think that the combination of these two types of studies is necessary, as they may help in elaborating more accurate, complete and sophisticated environmental and population health assessments.

On the other hand, visual sorting of microplastics is sometimes biased and quite difficult to perform when the product of the digestion is complex; since sometimes microplastics and natural small items can be very similar to the eyes even under a microscope. Moreover, eyesight becomes tired at some point during observation, which makes reliability even lower. For this reason, it is important to try and use complimentary methods such as polymer analyses. These challenges could be solved by using automated methods such as the one proposed by Primpke et al. (2017), consisting of performing an automated Focal Plane Array (FPA) which eliminates the observer bias, as well as factors such as tired eyesight or poor vision performance. However, as in our case, many laboratories do not have access to this technique. In spite of these handicaps, FT-IR is an easy-to-use, clean and cheap method for polymer analysis if we are willing to build our own reference library.

Conclusions

To summarize, the striped dolphin is frequently exposed to microplastics, mostly fibres. However, microplastic burden in their bodies seems relatively low for their body size. Hence, the concern is more about the chemical substances that can potentially damage dolphins' health and impact their population than about the quantity of microplastics physically present in their digestive systems. The use of striped dolphins as indicators of microplastic presence in pelagic biota at sea in the western Mediterranean is limited due to the difficulties in sampling and long processing time. Nevertheless, monitoring them is interesting and important in order to assess threats to their population. Both techniques used here revealed the importance of fibres probably coming from clothes and textiles. Increasing the amount of data about polymer composition is fundamental in order to precisely target the origin of most complicated residues and decrease their input to the sea. Despite having samples from a long period, most of the dolphins analysed here were from recent years, therefore limiting the detection of potential changes in long term periods. Nonetheless, in this study we establish a good baseline for future studies on this topic.

Supplementary material: table S7.1. Stripped dolphins' additional biometric parameters and location of stranding along the coast of the Valencian Community (Spain).

Dolphins	Municipality	Province	Year	Sex	Cause of death	Carcass condition	Length (cm)
Sc000315	El Saler	Valencia	2000	Female	Stranding	3	188
Sc011229B	Isla de Tabarca	Alicante	2001	Female	Stranding	2	100
Sc020427	Torrevieja	Alicante	2002	Female	Stranding	3	149
Sc060729	Sueca	Valencia	2006	Male	Stranding	3	96
Sc071027	Nules	Castellón	2007	Female	Stranding	2	201.8
Sc100814	Vilajoiosa	Alicante	2010	Male	Stranding	3	130
Sc110329	Altea	Alicante	2011	Male	Stranding	2	123
Sc120907	Alicante	Alicante	2012	Male	Stranding	2	108
Sc130415	Altea	Alicante	2013	Male	Stranding	3	93,6
Sc130808	Puzol	Valencia	2013	Male	Stranding	2	NA
Sc140201	Benidorm	Alicante	2014	Female	Stranding	3	199.5
Sc140818	Dénia	Alicante	2014	Female	Stranding	2	NA
Sc150213	Cullera	Valencia	2015	Male	Stranding	2	130
Sc150218	Sueca	Valencia	2015	Female	Stranding	2	192
Sc150317	Valencia	Valencia	2015	Male	Stranding	2	133
Sc150320B	Dénia	Alicante	2015	Female	Stranding	2	196
Sc160817	Dénia	Alicante	2016	Male	Stranding	3	120
Sc161201	El Saler	Valencia	2016	Female	Stranding	2	103
Sc161217	Alboraya	Valencia	2016	Male	Stranding	2	118
Sc161219	NA	Castellón	2016	Female	Stranding	2	198
Sc170118	Dénia	Alicante	2017	Male	Stranding	2	174

Supplementary material: table S7.1. (Continued) Stripped dolphins' additional biometric parameters and location of stranding along the coast of the Valencian Community (Spain).

Dolphins	Municipality	Province	Year	Sex	Cause of death	Carcass condition	Length (cm)
Sc170124	Dénia	Alicante	2017	Female	Stranding	2	130
Sc170216	Cullera	Valencia	2017	Male	Stranding	2	132
Sc170220A	Torrevieja	Alicante	2017	Male	Stranding	2	200
Sc170223	Orihuela	Alicante	2017	Male	Stranding	2	200
Sc170318	Vinaros	Alicante	2017	Male	Stranding	2	178
Sc170318	Santa Pola	Alicante	2017	Male	Stranding	3	178,8
Sc170401	Dénia	Alicante	2017	Female	Stranding	2	190
Sc170419	Oropesa	Castellón	2017	Female	Stranding	2	185
Sc170427	Perellonet	Valencia	2017	Male	Stranding	2	218
Sc170801	Dénia	Alicante	2017	Male	Stranding	2	184
SC170919	Sagunto	Valencia	2017	Female	Stranding	2	100
Sc89-02	Moncófar	Castellón	1989	Female	Stranding	2	206
Sc90-22	El Saler	Valencia	1990	Male	Stranding	2	204
Sc90-30	Torrevieja	Alicante	1990	Female	Stranding	3	192
Sc90-33	Pinedo	Valencia	1990	Male	Stranding	3	184
Sc90-64	Campello	Alicante	1990	Male	Stranding	3	202
Sc900715	Canet	Valencia	1990	Male	Stranding	2	167
Sc900828	Alboraya	Valencia	1990	Female	Stranding		201
Sc960104	Benidorm	Alicante	1996	Male	Stranding	2	192
Sc960215	Dénia	Alicante	1996	Female	Stranding	2	193
Sc970905	El Saler	Valencia	1997	Female	Stranding	2	95,5
ScMundomar-99	El Campello	Alicante	1999	Male	Aquarium Mundomar	2	168

NA = not available; Carcass condition: 1 (alive), 2 (freshly dead), 3 (starting to decompose), 4 (decomposed), 5 (putrefied, mummified, almost no skin, bones visible)

References

Aguilar, A. & Gaspari, S. 2012. Stenella coeruleoalba (Mediterranean subpopulation). The IUCN Red List of Threatened Species 2012: e.T16674437A16674052. http://dx.doi.org/10.2305/IUCN.UK.2012-1.RLTS.T16674437A16674052.en.

Association of plastics manufacturers, 2018. Plastics-the facts 2018. https://www.palsticseurope.org

Aznar F.J., Miguel-Lozano R., Ruiz B., Bosch de Castro A., Raga J.A., Blanco C., 2017. Long-term changes (1990 – 2012) in the diet of striped dolphins *Stenella coeruleoalba* from the western Mediterranean. *Marine Ecology Progress Series* 568:231-247. https://doi.org/10.3354/meps12063

Bakir A., Rowland S.J., Thompson R.C., 2014. Enhanced desorption of persistent organic pollutants from microplastics under simulated physiological conditions. *Environmental Pollution*, 185:16-23. https://doi.org/10.1016/j.envpol.2013.10.007

Bellas J., Martínez-Armental J., Martínez-Cámara A., Besada V., Martínez-Gómez C., 2016. Ingestion of microplastics by demersal fish from the Spanish Atlantic and Mediterranean coasts. *Marine Pollution Bulletin* 109(1):55-60. https://doi.org/10.1016/j.marpolbul.2016.06.026

Besseling E., Foekema E.N., Van Franeker J.A., Leopold M.F., Kühn S., Bravo-Rebolledo E.L., Heße E., Mielke L., IJzer J., Kamminga P., Koelmans A.A., 2015. Microplastic in a macro filter feeder: humpback whale *Megaptera novaeangliae*. *Marine Pollution Bulletin* 95(1):248-252. https://doi.org/10.1016/j.marpolbul.2015.04.007

Blanco C., Aznar J., Raga J.A., 1995. Cephalopods in the diet of the striped dolphin *Stenella coeruleoalba* from the western Mediterranean during an epizootic during 1990. *Journal of Zoology* 237: 151-158. https://doi.org/10.1111/j.1469-7998.1995.tb02753.x

BOE 301, 14th December of 2018, section 3, BOE-A-2018-17117, 2018. *Ministerio para la Transición Ecológica*, España. pp: 123041 - 123047.

Choy A.C., Robinson B.H., Gagne T.O., Erwin B., Firl E., Halden R.U., Hamilton J.A., Katija K., Lisin S.E., Rolsky C., Van Houtan K.S., 2019. The vertical distribution and biological transport of marine microplastics across the epipelagic and mesopelagic water column. *Scientific Reports* 9:7843. https://doi.org/10.1038/s41598-019-44117-2

Cole M., Lindequet P., Fileman E., Halsband C., Goodhead R., Moger J., Galloway T.S., 2013. Microplastic ingestion by zooplankton. *Environmental Science Technology* 47(12):6646-6655. https://doi.org/10.1021/es400663f

Curran E., Hernández-Milian G., Rogan E., Whooley, 2014. Common dolphins in the River Iee at Cork city. *Ireland Naturalists' Journal* 33(2): 142.

De la Torre L. and Albaigés J., 2016. Oil Pollution in Spanish waters. In: Carpenter A and Kostianoy AG (eds.) Oil Pollution in the Mediterranean Sea: part II. The Handbook of Environmental Chemistry 84: 13 - 49. Springer. https://doi.org/10.1007/978-3-030-11138-0

Dede A., Salman A., Arda M.T., 2016. Stomach contents of by-caught striped dolphins (*Stenella coeruleoalba*) in the Eastern Mediterranean Sea. *Journal of the Marine Biological Association of the United Kingdom* 96: 869-875. https://doi.org/10.1017/s0025315415001538

Domenech F., Aznar F.J., Raga J.A., Tomás J., 2019. Two decades of monitoring marine debris ingestión in loggerhead sea turtle, *Caretta caretta*, from the western Mediterranean. *Environmental Pollution* 244:367-378. https://doi.org/10.1016/j.envpol.2018.10.047

El turismo en la Comunidad Valenciana 2018, 2019. Estadístiques de turismo de la Comunitat Valenciana, *Generalitat Valenciana*.

Eriksen M., Lebreton L.C.M., Carson H.S., Thiel N., Moore C.J., Borrero J.C., Galgani F., Ryan P.G., Reisser J., 2014. Plastic pollution in the world's oceans: more than 5 trillion plastic pieces weighing over 250,000 tons afloat at sea. *Plos One*. https://doi.org/10.1371/journal.pone.0111913

European Chemicals Agency, https://echa.europa.eu, accessed in December 2017.

European Commission DG ENV, 2000. Towards the establishment of a priority list of substances for further evaluation of their role in endocrine disruption - preparation of a candidate list of substances as a basis for priority setting; Annex 15.

Eurostat, 2017. Tourism statistics. http://ec.europa.eu/eurostat/statistics-explained/index.php?title=Tourism_statistics. Accessed in June, 2018.

Forcada J., Aguilar A., Hammond P.S., Pastor X., Aguilar R., 1994. Distribution and numbers of striped dolphins in the western Mediterranean Sea after epizootic outbreak. *Marine Mammal Science* 10(2):137-150. https://doi.org/10.1111/j.1748-7692.1994.tb00256.x

Fossi M.C., Panti C., Guerranti C., Coppola D., Giannnetti M., Marsilli L., Minutoli R., 2012. Are baleen whales exposed to the threat of microplastics? A case study of the Mediterranean fin whale (*Balaenoptera physalus*). *Marine Pollution Bulletin* 64(11):2374-2379. https://doi.org/10.1016/j.marpolbul.2012.08.013

Fossi M.C., Coppola D., Baini M., Giannnetti M., Guerranti C., Marsilli L., Panti C., de Sabata E, Clò S, 2014. Large filter feeding marine organisms in the pelagic environment: the case studies of the Mediterranean basking shark (*Cetorrhinus maximus*) and fin whale (*Balaenoptera physalus*). Marine Environmental Research 100:17-24. https://doi.org/10.1016/j.marenvres.2014.02.002

Fossi M.C., Marsilli L., Baini M., Giannetti M., Coppola D., Guerranti C., Caliani I., Minutoli R., Lauriano G, Grazia-Finoia M, Rubegini F, Panigada S, Bérubé M, Urbán-Ramírez J, Panti C, 2016. Fin whales and microplastics: The Mediterranean Sea and the Sea of Cortez scenarios. *Environmental Pollution* 209:68-78. https://doi.org/10.1016/j.envpol.2015.11.022

Fossi M.C., Panti C., Baini M., Lavers J.L., 2018. A Review of Plastic-Associated Pressures: Cetaceans of the Mediterranean Sea and Eastern Australian Shearwaters as Case Studies. *Frontiers in Marine Science*, 5. https://doi.org/10.3389/fmars.2018.00173

Frias J.P.G.L., Sobral P., Ferreira A.M., 2010. Organic pollutants in microplastics from two beaches of the Portuguese coast. *Marine Pollution Bulletin* 60(11):1988-1992. https://doi.org/10.1016/j.marpolbul.2010.07.030

Gannier A., 2005. Summer distribution and relative abundance of Delphinids in the Mediterranean Sea. *Révue Ecologie (Terre Vie)* 60:223–238.

Gago J., Galgani F., Maes T., Thompson R.C., 2016. Microplastics in Seawater: Recommendations from the Marine Strategy Framework Directive Implementation Process. *Frontiers in Marine Science*, 3. https://doi.org/10.3389/fmars.2016.00219

Galloway T.S., Cole M. and Lewis C., 2017. Interactions of microplastics throughout the marine ecosystem. Nature Ecology and Evolution 1:0116. https://doi.org/10.1038/s41559-017-0116

GESAMP, 2015. Sources, fate and effects of microplastics in the marine environment: a global assessment. (Kershaw, P. J., ed.). (IMO/FAO/UNESCO-IOC/UNIDO/WMO/IAEA/UN/UNEP/UNDP Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection). Rep Stud GESAMP No. 90, 96 p.

Gewert B., Plassmann M.M., MacLeod M., 2015. Pathways for degradation of plastic polymers floating in the marine environment. *Environmental Science: Processes & Impacts* 17: 1513–1521. https://doi.org/10.1039/C5EM00207A

Gómez de Segura A., Crespo E.A., Pedraza P.S., Hammond P.S., Raga J.A., 2006. Abundance of small cetaceans in waters of the central Spanish Mediterranean. *Marine Biology* 150:149-160. https://doi.org/10.1007/s00227-006-0334-0

Gozalbes P., Jiménez J., Raga J.A., Esteban J.A., Tomás J., Gómez J.A., Eymar J., 2010. Cetáceos y tortugas marinas en la Comunitat Valenciana. 20 años de seguimiento. Col·lecció Treballs

Tècnics de Biodiversitat, 3. Consellería de Medio Ambiente, Agua, Urbanismo y Vivienda. *Generalitat Valenciana*. Valencia.

INE, Instituto Nacional de Estadística (2019). Available at: <www.ine.es> [Accessed on 29th october 2019].

Hanke G., Galgani F., Werner S., Oosterbaan L., Nilsson P., Fleet D., Kinsey S., Thompson R., Van Franeker J.A., Vlachogianni T., Palatinus A., Scoullos M., Veiga J.M., Matiddi M., Alcaro L., Maes T., Korpinen S., Budziak A., Leslie H., Gago J., Liebezeit G., 2013. Guidance on monitoring of marine litter in European Seas. *European Commission*. http://hdl.handle.net/10508/1649

Ivar do Sul J.A., Costa M.F., 2014. The present and future of microplastic pollution in the marine environment. *Environmental Pollution* 185:352-364. https://doi.org/10.1016/j.envpol.2013.10.036

Jambeck J.R., Geyer R., Wilcox C., Siegler T.R., Perryman M., Andrady A., Narayan R., Law K.L., 2015. Plastic waste inputs from land into the ocean. *Science* 347(6223):347. https://doi.org/10.1126/science.1260352

Jung M.R., 2018. Validation of ATR FT-IR to identify polymers of plastic marine debris, including those ingested by marine organisms. *Marine Pollution Bulletin* 127:704-716. https://doi.org/10.1016/j.marpolbul.2017.12.061

Karlsson T.M., Vethaak A.D., Almroth B.C., Ariese F., Velzen M., Hassellöv M., Leslie H.A., 2017. Screening for microplastics in sediment, water, marine invertebrates and fish: method development and microplastic accumulation. *Marine Pollution Bulletin* 122:403-408. https://doi.org/10.1016/j.marpolbul.2017.06.081

Liubartseva S., Coppini G., Lecci R., Clementi E., 2018. Tracking plastics in the Mediterranean: 2D Lagrangian model. *Marine Pollution Bulletin* 129:151–162. https://doi.org/10.1016/j.marpolbul.2018.02.019

Lusher A.L., Hernandez-Milian G., O'Brien J., Berrow S., O'Connor I., Officer R., 2015. Microplastic and macroplastic ingestion by a deep diving, oceanic cetacean: The True's beaked whale Mesoplodon mirus. *Environmental Pollution* 199:185–191. https://doi.org/10.1016/j.envpol.2015.01.023

Lusher A., Welden N., Sobral P., Cole M., 2016. Sampling, isolating and identifying microplastics ingested by fish and invertebrates. *Analytical Methods* 9:1346–60. https://doi.org/10.1039/C6AY02415G

Lusher A.L., Hernandez-Milian G., Berrow S., Rogan E., O'Connor I., 2018. Incidence of marine debris in cetaceans stranded and bycaught in Ireland: Recent findings and a review of historical knowledge. *Environmental Pollution* 232:467–476. https://doi.org/10.1016/j.envpol.2017.09.070

Meissner A.M., MacLeod C.D., Richard P., Ridoux V., Pierce G., 2012. Feeding ecology of striped dolphin, *Stenella coeruleoalba*, in the north-western Mediterranean Sea based on stable isotope analyses. *Journal of the Marine Biological Association of the United Kingdom* 92(8):1677 – 1687. https://doi.org/10.1017/S0025315411001457

Messinetti S., Mercurio S., Parolini M., Sugni M., Pennati R., 2018. Effects of polystyrene microplastics on early stages of two marine invertebrates with different feeding strategies. *Environmental Pollution* 237:1080-1087. https://doi.org/10.1016/j.envpol.2017.11.030

Moore M.J., van de Hoop J., Barco S.G., Costidis A.M., Gulland F.M., Jepson P.D. […], McLellan W.A., 2013. Criteria and definitions for serious injury and death of pinnipeds and cetaceans caused by anthropogenic trauma. *Diseases of Aquatic Organisms* 103:229-264. https://doi.org/10.3354/dao02566

Millot C., 1999. Circulation in the western Mediterranean Sea. *Journal of Marine Systems* 20:423 – 442. https://doi.org/10.1016/S0924-7963(98)00078-5

Nelms S.E., Galloway T.S., Godley B.J., Jarvis D.S., Lindeque P.K., 2018. Investigating microplastic trophic transfer in marine top predators. *Environmental Pollution* 238:999-1007. https://doi.org/10.1016/j.envpol.2018.02.016

Nelms, S.E., Barnett J., Brownlow A., Davison N.J., Deaville R., Galloway T.S., Lindeque P.K., Santillo D., Godley B.J, 2019. Microplastics in marine mammals stranded around the British coast: ubiquitous but transitory*? Nature Scientific Report*s 9:1075. https://doi.org/10.1038/s41598-018-37428-3

Neves D., Sobral P., Ferreira J.L., Pereira T., 2015. Ingestion of microplastics by comercial fish off the Portuguese coast. *Marine Pollution Bulletin* 101(1): 119-126. https://doi.org/10.1016/j.marpolbul.2015.11.008

Notarbartolo di Sciara G., Venturino M.C., Zanardelli M., Bearzi G., Borsani F.J., Cavalloini, B., 1993. Cetaceans in the Mediterranean Sea: distribution and sighting frequencies. *Bolletino di Zoologia* 60:131 – 138. https://doi.org/10.1080/11250009309355800

Primpke S., Lorenz C., Rascher-Friesenhausen R., Gerdts G., 2017. An automated approach for microplastics analysis using focal plane array (FPA) FTIR microscopy and image analysis. *Analytic Methods* 9:1499-1511. https://doi.org/10.1039/C6AY02476A

Ringelstein J., Pusineri C., Hassani S., Meynier L., Nicolas R., Ridoux V., 2006. Food and feeding ecology of the striped dolphin, *Stenella coeruleoalba*, in the oceanic waters of the north-east Atlantic. *Journal of the Marine Biological Association of the United Kingdom* 86(4):909–918. https://doi.org/10.1017/S0025315406013865

Schwacke L.H., Gulland F.M., White S., 2013. Sentinel Species in Oceans and Human Health. In: Laws E (eds) *Environmental Toxicology*, 503-528. Springer, New York, NY.

Simon-Sánchez L., Grelaud M., Garcia-Orellana J., Ziveri P., 2019. River deltas as hotspots for microplastic accumulation: the case study of the Ebro river. *Science of the Total Environment*, 687:1186 – 1196. https://doi.org/10.1016/j.scitotenv.2019.06.168

Spain. Ministry of Agriculture and Fishing, Nourishment and Environment, 2017. Technical Assistance in the implementation of the marine strategy frame directive: design, development and execution of the monitoring programmes. Monitoring of microparticles in beaches (BM-6).

Tomás J., Gozalbes P., Raga J.A., Godley B.J., 2008. Bycatch of loggerhead sea turtles: insights of 14 years of stranding data. *Endangered Species Research*, 5:161 – 169. https://doi.org/10.3354/esr00116

Unger B., Bravo-Rebolledo E., Deaville R., Gröne A., Lonneke L., Ijsseldijk, Leopold M.F., Siebert U., Spitz J., Wohlsein P., Herr H., 2016. Large amounts of marine debris found in sperm whales stranded along the North Sea coast in early 2016. *Marine Pollution Bulletin* 112: 134 – 141. https://doi.org/10.1016/j.marpolbul.2016.08.027

Webb H.K., Arnott J., Crawford R.J., Ivanova E.P., 2013. Plastic degradation and its environmental implications with special reference to Poly (ethylene terephthalate). *Polymers*, 5(1):1-18. https://doi.org/10.3390/polym5010001

8. Microplastics in *Lampanyctus crocodilus* (Myctophidae), a common lanternfish species from the Ibiza Channel (western Mediterranean)

Novillo-Sanjuan, O., Gallén, S., Raga, J. A., & Tomás, J. (Under Review). Microplastics in *Lampanyctus crocodilus* (Myctophidae), a common lanternfish species from the Ibiza Channel (western Mediterranean). *Animals*.

Abstract

Microplastics' presence in the pelagic environment is still largely unknown due to the difficulty of sampling in this environment. In this study, we quantify microplastics' exposure in a pelagic lanternfish species from the western Mediterranean, *Lampanyctus crocodilus*, which occupies an intermediate position in the marine food web. *L. crocodilus* were sampled in the Ibiza Channel and microplastics were extracted by digestion of their gastrointestinal systems. Almost half of the analysed lanternfish contained microplastics, mostly blue and black fibres (40.9% and 34.66%, respectively). In fishes with at least one microplastic, the median was 3 MPs/fish (CI 95% = 3.46 – 6.8); similar to other studies performed in other fish species in the area. Biometric parameters of fish, such as total length, sex and body condition, were not correlated with the number of microplastics. Data presented here contributes to quantify the severity of microplastic pollution in the pelagic environment and in a wild, non-commercial species.

Introduction

Microplastics have been documented in all kind of marine ecosystems, from the surface to the seafloor (Pham et al. 2014; Jamieson et al. 2019), from coastal environments to the open ocean (Pham et al. 2014; Jung et al. 2021). Some studies have calculated that around 2200-4000 t of plastic are released globally into the sea every year (Kaandorp et al. 2020; de la Fuente et al. 2021), while other studies have calculated even higher amounts, 200000 t per year, and that by 2040 this quantity could reach 500000 t per year (Boucher and Billard, 2020). It is estimated that between 21% and 54% of the global microplastic particles floating at sea, and that between 5% and 10% of their global mass, are expected to be found in the Mediterranean Sea (van Sebille et al. 2015). The Mediterranean is especially vulnerable to the accumulation of any kind of contaminants due to its limited water exchange with the oceans and the great anthropogenic pressure it is exposed to.

Despite the growing knowledge on this matter, information about microplastics' occurrence below the water surface and in the food web is limited. Even positive buoyant microplastics may eventually sink towards the ocean floor, helped by biofouling and by the creation of biofilms (Kaiser et al. 2017). Some model projections predict that only 1% of microplastics remain at surface; therefore, the ocean depths could be a potential reservoir that could not only be formed by sinking microplastics, but also from the fragmentation of bigger plastic items that are already present on the seafloor (Pham et al. 2014). Fish species have been hypothesized as the other big reservoir, together with the sea surface (van Sebille et al. 2015). Furthermore, they can incorporate microplastics into the food web while drinking and foraging on organisms that had previously ingested microplastics (Farrell and Nelson, 2013; Setälä et al. 2014, Nelms et al. 2018, Wang et al. 2019; Novillo-Sanjuan et al. 2020, Roch et al. 2020). Microplastics might be potentially harmful to organisms by leaking toxic substances, such as persistent organic pollutants (POPs), along their transit through their gastrointestinal tracts (Rodrigues et al. 2019).

Up to date, several animal species from different parts of the Mediterranean have been studied for microplastic content (table 8.2). Lanternfish species are great candidates to gather data about microplastics in the Mediterranean bathypelagic environment. Here we studied microplastic frequency and abundance in a non-commercial, wild lanternfish species present in the western Mediterranean: the jewel lanternfish (*Lampanytus crocodilus,* Risso, 1910; Myctophidae family). They play a key role in carbon transfer from the seawater surface layer to the bottom of the sea by vertically migrating on a daily basis (Fannelli et al. 2014). Besides, they have been described as important prey species for the striped dolphin (*Stenella coeruleoalba*), the most abundant cetacean species in the western Mediterranean (Gómez de Segura et al. 2006; Aznar et al. 2017), and could contribute to pollutant transfer to this species.

In the present study we aim to quantify microplastics in the gastrointestinal tracts of a bathypelagic lanternfish species (*Lampanyctus crododilus*) and to increase the knowledge about microplastic occurrence in an understudied part of the water column (the bathypelagic environment) and about the potential role of lanternfish in microplastics distribution.

Materials and methods

Sampling and study area

In total, 94 lanternfish (*Lampanyctus crocodilus*) were analysed for microplastics. The fishes were bycaught on the 20th of June 2017, by a trawling fishing vessel the target species of which were prawns. Fishing operation was carried out off the continental shelf, in between the Valencian Community coast (East peninsular Spain) and the Balearic Islands; starting 39°06'13"N 00°24'00"E (406 braces) and ending at 38°51'60"N 00°34'52"E (402 braces), trawling at approximately 300 m depth (figure 8.1). Myctophids were weighed, measured and stored at -20 ºC.

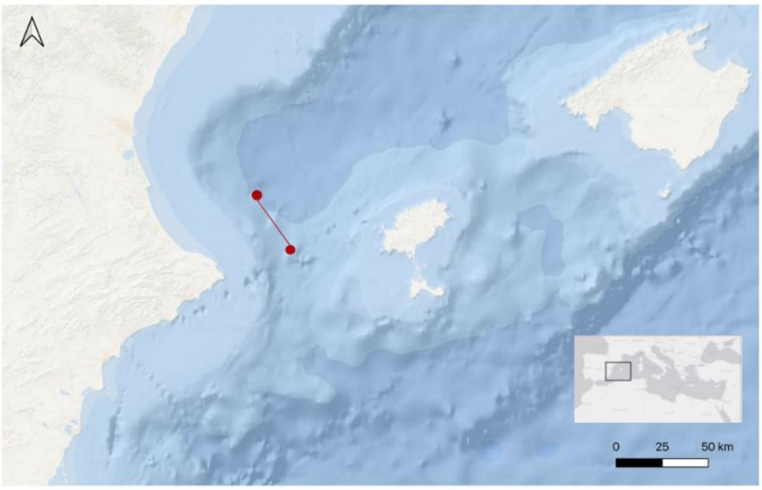


Figure 8.1. Trajectory from start to finish (indicated by the red line) of the fishing vessel used for sampling of Myctophids around the Ibiza channel (Western Mediterranean). Exact coordinates of red dots are indicated in the main text.

Laboratory procedure

Myctophids were thawed at room temperature and necropsied under a dissecting microscope (Leica MZ APO, 8 – 80x) with stainless steel scalpels and tweezers. For the extraction of microplastics, we followed the methods used by Lusher et al. (2016) and Foekema et al. (2013), with few adaptations (see below). Briefly, all the gastrointestinal tracts were removed, opened and observed in order to collect microplastics and other gut contents. Once diet and parasites were separated for further studies, the remains, including the gastrointestinal tissue, were digested in KOH 10% 1:3 v/v for a week at room temperature. After digestion, samples were filtered under vacuum through a Büchner system equipped with Whatman GC/F borosilicate glass microfibre filters (1.2 μm pore size). These filters were then dried for 24h at 60ºC and, after that, they were carefully observed under the same dissecting microscope where dissections took place. Microplastics found were separated and classified into categories of size, shape (fibre, fragment, film or pellet) and colour. In order to ensure that we were observing anthropogenic material, microplastic items were also observed under microscope (Leica DMR), in which it is easier to observe characteristic surfaces of virgin plastic and cracked plastic, threads, and filaments (Wang et al. 2017). Additionally, the hot needle test was to see if the studied items melted under the heat, which would indicate that the item is made of plastic (Silva et al. 2018).

Contamination control

Regarding the ubiquity of microplastics in the environment, a series of measures were taken to minimise potential contamination of the samples in the workplace. Plastic materials were avoided as much as possible in all procedures. All materials used here were made either of stainless steel or glass; and they were thoroughly cleaned with deionized water and ethanol 70% prior to analysis. The sponges used to clean were always made of the same bright yellow colour, so as to quickly identify potential contamination from the sponges' fibres. The potassium hydroxide solution (KOH 10%) was filtered through GC/F filters before using it for the digestion of biological material. Additionally, all filtrations were performed under a type I laminar flow cabinet with positive pressure in order to prevent the introduction of external contamination through air in the workspace.

Procedural blanks were prepared in order to monitor external contamination. Clean GC/F filters were exposed to the same environments and for the same amount of time as the real samples, from the start of the necropsy to the final observation. Afterwards, they were also observed under the same dissecting microscope to quantify potential microplastics present in the workspace. Microplastics found were subtracted from the samples accordingly.

In spite of all these measures, contamination control was not possible on-board during fishing operations due to the opportunistic nature of the sampling. Sampling took advantage of fishing campaigns and fishermen could not be bothered during work. However, fish were immediately frozen at -20ºC onboard and processed in the lab, which was clean and where procedural blanks were already present.

Statistical analyses

Statistical analyses were calculated in Rstudio (1.2.5033) and data visualization was carried out using the R package ggplot2 (Wickham, 2016). Confidence intervals for the mean and medians of microplastics per individual (both in lantern fish and striped dolphins), were calculated in Qpweb (version 1.0.15; Reiczigel et al. 2019) by bootstrapping 10000 replicates. In order to assess the body condition of the fishes, the Fulton's K condition factor was calculated with the following equation (Froese, 2006):

$$K = \frac{weight}{length^3} \times 100;$$

where length is the total length of the fish. Both weight and length refer to measures taken before the fish were frozen. The closer to 1, the better body condition has the fish and vice versa. A Pearson's Correlation test was calculated to check whether microplastic amount was correlated with body condition (Fulton's K).

Results

Biometric parameters

A total of 94 lanternfishes (*L. crocodilus*) were examined in search of microplastics. On average, fish measured 16.83 ± 1.46 cm (mean total length± SD) following a normal distribution. All lanternfishes in this study were adult specimens, according to previous literature (Stefanescu and Cartes 1992; Fannelli et al. 2014). Sex of the fish was strongly biased: 76.92% were females, 7.69% males and 15.38% undetermined, so correlations between microplastics' content and sex were not calculated. Fulton's K body condition factor was 0.526 ± 0.06.

Microplastic content and characteristics

More than the half of *L. crocodilus* (59.79%) did not show microplastics, while 40.21% of myctophids presented at least one microplastic. In total, 185 microplastics items were identified. Nevertheless, even among those which presented microplastics, the number of items was generally close to 1 and, therefore, data showed a strongly right-skewed distribution. In fishes with at least one microplastic, median was 3 MPs/fish (CI 95% = 3.46 – 6.8).

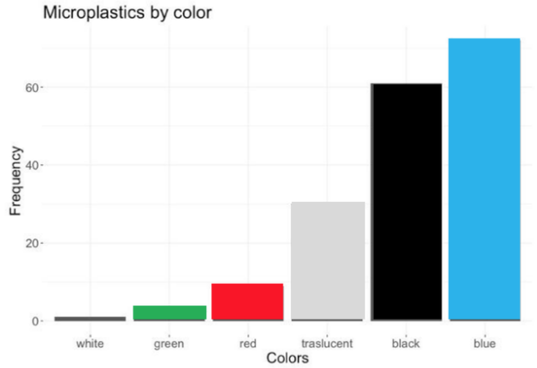
Table 8.1. Mean, median, range and confidence intervals of the microplastics found in fishes in this study, together with their mean Fulton's K body condition index.

	All myctophids	**Myctophids with microplastics**
Mean (MPs/fish)	1.907	4.744
95% CI for the mean	1.26 – 2.92	3.44 – 6.87
Median (MPs/fish)	0	3
95% CI for the mean	1.27 - 2.97	3.46 - 6.8
Range (MPs/fish)	0 - 23	1 - 23
Mean Fulton's K	0.5268	0.5097

The most frequent microplastic colour was light blue (40.9%), followed by black (34.66%), translucent (17.0%), red (5.11%), green (1.7%) and white items (0.57%) (figure 2, a). As figure 2b shows, most items measured in between 1 and 3 mm (24.59%), followed by 3 – 4 mm (22.46%), 0.6 – 1 mm (19.25%), 0.2 – 0.6 mm (15.5%), 4 – 5 mm (13.37%) and <0.2 mm (4.81%). In addition, almost all microplastics were fibres (97.75%, figure 3) and only 2.25% were fragments. No pellets or primary microplastics were found in the analyzed fishes.

Factors affecting microplastic ingestion

Fish length and number of microplastics found were not correlated ($r = -0.058$, $p > 0.05$), nor when considering only fish that ingested microplastics ($r = 0.08$, $p > 0.05$). There was no significant correlation between body condition (Fulton's K) and amount of ingested microplastics neither (Pearson's correlation product = - 0.175).



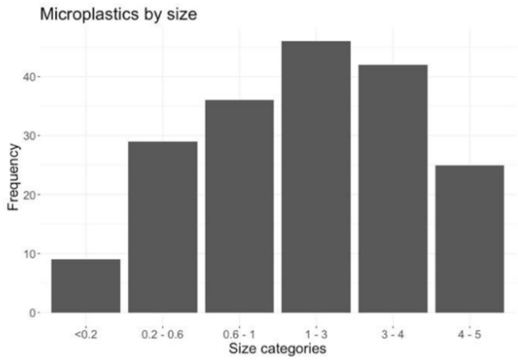


Figure 8.2. A) Colour of microplastics found in Mediterranean lanternfish in ascent order of frequency and b), frequency of microplastics found in each size category.

Discussion

Microplastics in lanternfish species

Frequency of occurrence found in this research is similar to other studies performed elsewhere in the Mediterranean with different species; including the north-western Mediterranean (Bellas et al. 2016; Ríos-Fuster et al. 2019), the Ligurian Sea (Capone et al. 2020), the eastern Mediterranean (Güven et al. 2017) and the Adriatic and Ionian Seas (Anastasopoulou et al. 2018 and Digka et al. 2018, table 8.2). In comparison to a study on Atlantic mesopelagic fish (Wieczorek et al. 2018), our frequency of occurrence was lower (40% in contrast to 73%). However, myctophids from the North Atlantic and in the Indian ocean showed lower microplastic content (frequency of occurrence: 0 – 22% and 2%, respectively) and, specifically, no microplastics were found in *L. crocodilus*, although the N was very low (n = 2; Lusher et al. 2016; Bernal et al. 2020). Previous studies on Mediterranean myctophids found a far lower frequency of occurrence (0% – 5.8%, migratory and non-migratory species, respectively; Romeo et al. 2016; Bernal et al. 2015). The plastics found in those studies were mainly clear and of the size of copepods and other lanternfishes' prey, suggesting that confounding microplastics with food might occur. Regarding colour composition, it varies widely across studies. Here, the most frequent colours observed were blue and black, as in some studies carried out with other Mediterranean fish species (see table 8.2) and in other oceans (Woodall et al. 2014; Lusher et al. 2014; Neves et al. 2015; Hernández-González et al. 2018 and Barboza et al. 2020). Although there is variation, blue and black plastics might be the most prevalent colours in some regions due to their frequency of use and possibly due to a higher resistance to colour degradation. Lanternfish in our study fed on zooplanktonic species, such as euphausiids and mysids, which agrees with previous studies (Froese and Pauly, 2000; Fanelli et al. 2014; Valls et al. 2014). These zooplanktonic species are not similar to microplastics found in the gastrointestinal tracts of the examined fish, neither in colour nor in shape, so potential selective feeding of microplastics seems unlikely. The question of whether fish selectively feed on these items, remains unclear. Besides, microplastics could also be accidentally ingested while swimming, drinking and even through the gills. Interestingly, fish that feed predominantly in a chemosensitive way, may be able to avoid foraging on microplastics, while visually oriented fish could eat more of them when microplastics resemble their prey (Roch et al. 2020). *L. crocodilus* is known to be mainly a visual predator (Bozzano et al. 2007), so microplastic selectivity should be low regarding their dissimilarity to their diet. In addition, when food is not abundant, fish seem to be prone to consume more microplastics, probably driven by an opportunistic feeding strategy developed in an environment with low food availability. Therefore, visually oriented fish in environments with low food availability could be more vulnerable to microplastics' exposure (Roch et al. 2020).

Most of the microplastics found here were fibres (figure 3), similarly as in most studies about microplastics in the Mediterranean Sea (table 8.2). Ríos-Fuster et al. (2019) also found mostly fibres in four fish species (*Trachurus mediterraneus*, *Sardina pilchardus*, *Engraulis encrasicolus* and *B. boops*, table 8.2*)*; and showed that species closer to the East Spain's coastline tended to present higher amounts of microplastics than those sampled close to the Balearic Islands. This ubiquity of fibers and filaments, maybe due to an inefficient waste-water treatment, an intensive use of washing machines (Browne et al. 2011; Ríos-Fuster et al. 2019) and to the use and improper disposal of fishing gear (Montarsolo et al. 2018). Moreover, species examined by Ríos-Fuster et al. (2019) belong to a similar trophic level (Froese et al. 2020) and have similar amounts of plastic than lanternfish in our study; suggesting that differences in microplastic content among species could be caused mostly by abiotic factors, such as sea currents, distance to point sources and wind regime, rather than by feeding ecology (Reissner et al. 2015; van Sebille et al. 2015; Zhang, 2017; Alava et al. 2020).

Microplastics and fish fitness

In our study, number of microplastic items per individual was not related neither to body condition nor to total length of the fish, so these variables could not be used as a predictor of microplastic ingestion risk. In our case, weaker, smaller fish do not seem more prone to microplastic ingestion than bigger, fitter fish; as has been shown in other studies (Kazour et al. 2019; Galafassi et al. 2021). When looking at fish size, we find cases of both bigger fish having more microplastics (Filgueiras et al. 2020), or bigger fish having less microplastics (Compa et al. 2018); and cases in which fish weight and length are not related at all with microplastic content (Barboza et al. 2020, present study). In either case, it is important to gather more evidence about how environmentally relevant concentrations of microplastics could be affecting fish development and survival. Overall fitness in wild fish will depend on environmental quality of their surroundings, predator pressure and exposure to infections, among other factors.

Potential transfer of other contaminants from microplastics to the organism, is of concern as well. Exposure to these persistent organic pollutants could increase their bioaccumulation capacity. Rochman et al. (2014) found a positive correlation between polybrominated flame retardants (PBDEs) and plastic amount in the water where myctophids inhabited, although this relationship did not exist for other pollutants such as polychlorinated biphenyls (PCBs) and alkylphenols. However, up to date, some studies suggest that transfer of these contaminants to biota is scarce and can be neglected (Herzke et al. 2016; Koelmans et al. 2016; Lohman, 2017; Rodrigues et al. 2019). In fact, in some experimental setups, microplastic pollution did not affect fish fitness and survival significantly (Müller et al. 2020).

Influence of the environment in microplastic concentration in lanternfishes

Myctophids perform extensive vertical nychthemereal migrations; however, information about them is only collected from fishing grounds of limited depth (Stefanescu and Cartes, 1992; Fanelli et al. 2013). According to observations and modelling approaches, microplastics tend to accumulate in subsurface waters and on the sea bottom (Reissner et al. 2015; van Sebille et al. 2015; Choy et al. 2019; de la Fuente et al. 2021), specially between 200 – 600m (Choy et al. 2019) where lantern fish feed (Froese and Pauly, 2000). The vertical migration performed by these fish could be mimicking the biological whale pump (see

Roman and McCarthy, 2010; Roman et al. 2014; Savoca et al. 2021), besides cycling nutrients, they would contribute to the vertical circulation of microplastics throughout the water column and could introduce these items in the marine food web, making them bioavailable for longer periods of time. For instance, microplastics have also been found in striped dolphins in the area (Novillo et al. 2020). Striped dolphins prey on *L. crocodilus* (Aznar et al. 2017) and, therefore, these fish could be contributing to microplastic ingestion of some predators. Nevertheless, experimental studies on food transfer between species are needed to assess whether microplastic bioaccumulation could take place.

On the other hand, microplastic concentration may vary among seasons (Cincinelli et al. 2019; Macías et al. 2019; de la Fuente et al. 2021), and therefore, studies performed in different times of the year or with different oceanic conditions (up-welling, down-welling, fronts, winds, currents, etc.) would be of big interest to understand microplastic concentration, distribution and circulation at sea. Litter tends to accumulate during the summer months in the study area, when there is higher tourist activity (Macías et al. 2019). In consequence, an increase in sinking microplastics across the pelagic environment in this season is also expected.

In the western Mediterranean, microplastic concentrations are expected to be lower than in the eastern Mediterranean due to differences in water circulation and topography. The area covered in this study is connected to the Atlantic Ocean through the Gibraltar Strait, and it is located in a wide gulf, so there is significant water exchange and water flow, especially at surface level. Also, there is a north-east surface current that could hinder potential accumulation, together with the lack of physical barriers. These oceanographic characteristics could be a reason to explain the low value of microplastic content per fish found in this study.

Conclusions

Lanternfish species represent a very important proportion of the pelagic biomass; they are clue in carbon transfer from the deep sea to the epipelagic environment. However, little is known about how microplastics could be affecting them. *L. crocodilus* in this study contain low microplastics' amount per fish, although frequency of occurrence is relatively

Table 8.2. Mean microplastics (MPs) per individual ± standard deviation in demersal and pelagic species in different areas of the Mediterranean Sea, along with their main characteristics.

Area	**Species**	**Habitat**	**Mean ± SD MPs/individual**	**% fish with MPs**	**Most common shape**	**Most common colour**	**Reference**
W Med. (E Spain)	*Mullus barbatus**	Demersal	1.9 ± 1.29	10 – 33%	Fibers	Black	Bellas et al. 2016
Central Med. Sea	*Electrona risso, Hygophum benoiti, Myctophum punctatum*	Pelagic	1.09 ± 0.30 4.10 ± 3.08 1.91 ± 0.55	2.7%	Small microplastics	Hyaline	Romeo et al. 2016
Turkey	28 species	Demersal and pelagic	2.36	41%	Fibers	Blue	Güven et al. 2017
Balearic Sea (Spain)	*M. surmuletus*	Demersal	0.42 ± 0.04	27.3%	Filament	Blue	Alomar et al. 2017a
Balearic Sea (Spain)	*Galeus melastomus*	Demersal	0.34 ± 0.07	16.8%	Filament	Transparent	Alomar and Deudero, 2017b

Table 8.2. (Continued). Mean microplastics (MPs) per individual ± standard deviation in demersal and pelagic species in different areas of the Mediterranean Sea, along with their main characteristics.

Area	Species	Habitat	Mean ± SD MPs/individual	% fish with MPs	Most common shape	Most common colour	Reference
Spanish Med.	*Sardina pilchardus* and *Engraulis encrasicolus*	Pelagic	0.18 ± 0.20 (SE)	14.8%	Fibers	Blue	Compa et al. 2018
N Ionian	*S. pilchardus, P. erythrinus* and *M. barbatus*	Pelagic	0.8 ± 0.2 0.8 ± 0.2, 0.5 ± 0.2	47.2%, 42.1%, 32%.	Fragments	Blue	Digka et al. 2018
NE Ionian Sea, N Adriatic Sea	*Chelon auratus, M. barbatus, M. surmuletus, P. erythrinus, Sparus aurata, S. pilchardus, Solea solea.*	Demersal and pelagic	6.7 ± 3.5 2.5 ± 0.2 1.7 ± 0.2	40% - 87% -	–	–	Anastasopoulou et al. 2018
W Med. (Spain)	*Boops boops*	Pelagic	1.68 ± 0.31 0.50 ± 0.14 0.53 ± 0.14	46%	Fragments	Blue	García-Garín et al. 2019

Table 8.2. (Continued). Mean microplastics (MPs) per individual ± standard deviation in demersal and pelagic species in different areas of the Mediterranean Sea, along with their main characteristics.

Area	**Species**	**Habitat**	**Mean ± SD MPs/individual**	**% fish with MPs**	**Most common shape**	**Most common colour**	**Reference**
Adriatic Sea, N Thyrrenian and Ionian Sea	*M. barbatus* and *Merluccius merluccius* .	Demersal	0 – 1.75	23.3%	Fibers	Blue	Giani et al. 2019
E Med. (Lebanon)	*E. encrasicolus*	Pelagic	2.9 ± 1.9	83.4%	Fragments	Blue	Kazour et al. 2019
W Med. (Spain)	*Boops boops, E. encrasicolus, S. pilchardus* and *T. mediterraneus*	Pelagic	0 ± 0 - 1.22 ± 2.08	28%	Fibers	Blue	Ríos-Fuster et al. 2019
Thyrrenian Sea	*Pagellus spp.*	Demersal	Not specified.	10.25%	Fibers	Black	Savoca et al. 2019
NW Med. (Ligurian Sea)	*E. encrasicolus*	Pelagic	0.34 ± 0.29 – 0.12 ± 0.12 fibres/ind^{-1}	30 – 40%	Fibers	Blue and black	Capone et al. 2020
SE Mediterranean (Egypt)	*Caranx crysos, Liza aurata, Signus rivulatus* and *Epinephelus caninus*	Demersal	8.6 ± 1.52 - 2 ± 2.64	–	Fibers	Blue	Sayed et al. 2021*

Table 8.2. (Continued). Mean microplastics (MPs) per individual ± standard deviation in demersal and pelagic species in different areas of the Mediterranean Sea, along with their main characteristics.

Area	Species	Habitat	Mean ± SD MPs/individual	% fish with MPs	Most common shape	Most common colour	Reference
Central Med.(Italy)	*T. trachurus*	Pelagic	112.86 ± 38.93	90.6%	Filament		Chenet et al. 2021
SW Med. (Mar Menor)	*Sparus aurata*	Demersal	20.11 ± 2.94 MP kg^{-1}	100%	Fibers	White	Bayo et al. 2021
W Med. (E Spain)	*Lampanyctus crocodilus*	Pelagic	1.907 ± 4.023	40.21%	Fibers	Blue	This study

*When the reference included specimens from other seas and ocean basins, only specimens from the Mediterranean were taken into account.

higher when compared to myctophids from other studies. The presence of microplastics in organisms that perform vertical migrations from the benthic and pelagic environment to the photic zone could also be indication that microplastics in the ocean can be recovered from the sea bottom and being made bioavailable for longer than expected in the oceanic water column.

References

Alava, J.J., 2020. Modeling the Bioaccumulation and Biomagnification Potential of Microplastics in a Cetacean Foodweb of the Northeastern Pacific: A Prospective Tool to Assess the Risk Exposure to Plastic Particles. *Frontiers in Marine Science* 7, 566101. https://doi.org/10.3389/fmars.2020.566101

Alomar, C., Sureda, A., Capó, X., Guijarro, B., Tejada, S., & Deudero, S., 2017a. Microplastic ingestion by *Mullus surmuletus* Linnaeus, 1758 fish and its potential for causing oxidative stress. *Environmental Research*, 159, 135-142. https://doi.org/10.1016/j.envres.2017.07.043

Alomar, C., & Deudero, S., 2017b. Evidence of microplastic ingestion in the shark *Galeus melastomus* Rafinesque, 1810 in the continental shelf off the western Mediterranean Sea. *Environmental Pollution*, 223, 223-229. https://doi.org/10.1016/j.envpol.2017.01.015

Anastasopoulou, A., Mytilineou, C., Smith, C. J., Papadopoulou, K. N., 2013. Plastic debris ingested by deep-water fish of the Ionian Sea (Eastern Mediterranean). *Deep Sea Research Part I: Oceanographic Research Papers*, 74, 11-13. https://doi.org/10.1016/j.dsr.2012.12.008

Aznar, F.J., Míguez-Lozano, R., Ruiz, B., de Castro, A., Raga, J.A., Blanco, C., 2017. Long-term changes (1990-2012) in the diet of striped dolphins *Stenella coeruleoalba* from the western Mediterranean. *Marine Ecolology Progress Series* 568, 231–247. https://doi.org/10.3354/meps12063

Baini, M., Fossi, M.C., Galli, M., Caliani, I., Campani, T., Finoia, M.G., Panti, C., 2018. Abundance and characterization of microplastics in the coastal waters of Tuscany (Italy): the application of the MSFD protocol in the Mediterranean Sea. *Marine Pollution Bulletin* 133, 543 – 552. https://doi.org/10.1016/j.marpolbul.2018.06.016

Barboza, L. G. A., Lopes, C., Oliveira, P., Bessa, F., Otero, V., Henriques, B., Raimundo, J., Caetano, M., Vale, C., Guilhermino, L., 2020. Microplastics in wild fish from North East Atlantic Ocean and its potential for causing neurotoxic effects, lipid oxidative damage, and human health risks associated with ingestion exposure. *Science of the total environment* 717, 134625. https://doi.org/10.1016/j.scitotenv.2019.134625

Barlow, J., Kahru, M., Mitchell, B.G., 2008. Cetacean biomass, prey consumption, and primary production requirements in the California Current ecosystem. *Marine Ecology Progress Series* 371, 285 – 295. https://doi.org/10.3354/meps07695

Bayo, J., Rojo, D., Martínez-Baños, P., López-Castellanos, J., Olmos, S., 2021. Commercial Gilthead Seabream (*Sparus aurata* L.) from the Mar Menor Coastal Lagoon as Hotspots of Microplastic *Accumulation in the Digestive System. International Journal of Environmental Research and Public Health*, 18(13), 6844. https://doi.org/10.3390/ijerph18136844

Bellas, J., Martínez-Armental, J., Martínez-Cámara, A., Besada, V., Martínez-Gómez, C., 2016. Ingestion of microplastics by demersal fish from the Spanish Atlantic and Mediterranean coasts. *Marine Pollution Bulletin* 109, 55–60. https://doi.org/10.1016/j.marpolbul.2016.06.026

Bernal, A., Olivar, M. P., Maynou, F., and de Puelles, M. L. F., 2015. Diet and feeding strategies of mesopelagic fishes in the western Mediterranean. *Progress in Oceanography*, 135, 1-17. https://doi.org/10.1016/j.pocean.2015.03.005

Bernal, A., Toresen, R., and Riera, R., 2020. Mesopelagic fish composition and diets of three myctophid species with potential incidence of microplastics, across the southern tropical gyre. *Deep Sea Research Part II: Topical Studies in Oceanography*, 179, 104706. https://doi.org/10.1016/j.dsr2.2019.104706

Boucher, J., Billard, G., 2020. The Mediterranean: Mare Plasticum. Gland, Switzerland: IUCN. Pp 38

Bozzano, A., Pankhurst, P. M., and Sabatés, A., 2007. Early development of eye and retina in lanternfish larvae. *Visual neuroscience*, 24(3), 423-436. https://doi.org/10.1017/S0952523807070484

Browne, M. A., Crump, P., Niven, S. J., Teuten, E., Tonkin, A., Galloway, T., Thompson, R., 2011. Accumulation of microplastic on shorelines woldwide: sources and sinks. *Environmental Science & Technology*, 45, 9175-9179. https://doi.org/10.1021/es201811s

Capone, A., Petrillo, M., Misic, C., 2020. Ingestion and elimination of anthropogenic fibres and microplastic fragments by the European anchovy (*Engraulis encrasicolus*) of the NW Mediterranean Sea. *Marine Biology* 167, 1-15. https://doi.org/10.1007/s00227-020-03779-7

Chenet, T., Mancia, A., Bono, G., Falsone, F., Scannella, D., Vaccaro, C., ... Pasti, L., 2021. Plastic ingestion by Atlantic horse mackerel (*Trachurus trachurus*) from central Mediterranean Sea: A potential cause for endocrine disruption. *Environmental Pollution*, 117449. https://doi.org/10.1016/j.envpol.2021.117449

Choy, C.A., Robison, B.H., Gagne, T.O., Erwin, B., Firl, E., Halden, R.U., Hamilton, J.A., Katija, K., Lisin, S.E., Rolsky, C., S. Van Houtan, K., 2019. The vertical distribution and biological transport of marine microplastics across the epipelagic and mesopelagic water column. *Science Reports* 9, 7843. https://doi.org/10.1038/s41598-019-44117-2

Cincinelli, A., Martellini, T., Guerranti, C., Scopetani, C., Chelazzi, D., Giarrizzo, T., 2019. A potpourri of microplastics in the sea surface and water column of the Mediterranean Sea. *TrAC Trends in Analytical Chemistry* 110, 321 – 326. https://doi.org/10.1016/j.trac.2018.10.026

Clarke, M.R., 1996. Cephalopods as prey. III. Cetaceans. *Philosophical Transactions of the Royal Society of London Series B: Biological Sciences* 351(1343), 1053-1065.https://doi.org/10.1098/rstb.1996.0093

Cole, M., Lindeque, P., Fileman, E., Halsband, C., Goodhead, R., Moger, J., Galloway, T.S., 2013. Microplastic ingestion by zooplankton. *Environmental science & technology*, 47(12), 6646-6655. https://doi.org/10.1021/es400663f

Compa, M., Ventero, A., Iglesias, M., Deudero, S., 2018. Ingestion of microplastics and natural fibres in *Sardina pilchardus* (Walbaum, 1792) and *Engraulis encrasicolus* (Linnaeus, 1758) along the Spanish Mediterranean coast. *Marine Pollution Bulletin* 128, 89-96. https://doi.org/10.1016/j.marpolbul.2018.01.009

de la Fuente, R., Drótos, G., Hernández-García, E., Jópez, C., van Sebille, E., 2021. Sinking microplastics in the water column: simulations in the Mediterranean Sea. *Ocean Science*, 17, 431 – 453. https://doi.org/10.5194/os-17-431-2021

de Vries, A. N., Govoni, D., Árnason, S. H., Carlsson, P., 2020. Microplastic ingestion by fish: Body size, condition factor and gut fullness are not related to the amount of plastics consumed. *Marine Pollution Bulletin* 151, 110827.

Digka, N., Tsangaris, C., Torre, M., Anastasopoulou, A., Zeri, C., 2018. Microplastics in mussels and fish from the Northern Ionian Sea. *Marine Pollution Bulletin* 135, 30-40. https://doi.org/10.1016/j.marpolbul.2018.06.063

Fanelli, E., Papiol, V., Cartes, J.E., Rodriguez-Romeu, O., 2014. Trophic ecology of Lampanyctus crocodilus on north-west Mediterranean Sea slopes in relation to reproductive cycle and environmental variables. *Journal of Fish Biology* 84, 1654–1688. https://doi.org/10.1111/jfb.12378

Farrell, P., Nelson, K., 2013. Trophic level transfer of microplastic: *Mytilus edulis* (L.) to *Carcinus maenas* (L.). *Environmental Pollution* 177, 1 – 3. https://doi.org/10.1016/j.envpol.2013.01.046

Filgueiras, A. V., Preciado, I., Cartón, A., Gago, J., 2020. Microplastic ingestion by pelagic and benthic fish and diet composition: a case study in the NW Iberian shelf. *Marine Pollution Bulletin* 160, 111623. https://doi.org/10.1016/j.marpolbul.2020.111623

Foekema, E. M., De Gruijter, C., Mergia, M. T., van Franeker, J. A., Murk, A. J., Koelmans, A. A., 2013. Plastic in North Sea fish. *Environmental science & technology* 47, 8818-8824. https://doi.org/10.1021/es400931b

Froese, R., 2006. Cube law, condition factor and weight–length relationships: history, meta-analysis and recommendations. *Journal of Applied Ichthyology* 22(4), 241-253. https://doi.org/10.1111/j.1439-0426.2006.00805.x

Froese, R., Pauly, D., (eds.), 2000. FishBase 2000, concepts, design and data sources. ICLARM Contrib. No.1594. International Center for Living Aquatic Resources Management (ICLARM). Los Banos, Laguna, Philippines. pp 344. ISBN: 971-8709-99-1

Galafassi, S., Campanale, C., Massarelli, C., Uricchio, V. F., Volta, P., 2021. Do Freshwater Fish Eat Microplastics? A Review with A Focus on Effects on Fish Health and Predictive Traits of MPs Ingestion. *Water* 13(16), 2214. https://doi.org/10.3390/w13162214

Garcia-Garin, O., Vighi, M., Aguilar, A., Tsangaris, C., Digka, N., Kaberi, H., Borrell, A., 2019. *Boops boops* as a bioindicator of microplastic pollution along the Spanish Catalan coast. *Marine Pollution Bulletin*, 149, 110648. https://doi.org/10.1016/j.marpolbul.2019.110648

Giani, D., Baini, M., Galli, M., Casini, S., Fossi, M. C., 2019. Microplastics occurrence in edible fish species (*Mullus barbatus* and *Merluccius merluccius*) collected in three different geographical sub-areas of the Mediterranean Sea. *Marine Pollution Bulletin* 140, 129-137.

Gómez de Segura, A., Crespo, E.A., Pedraza, S.N., Hammond, P.S., Raga, J.A., 2006. Abundance of small cetaceans in waters of the central Spanish Mediterranean. *Marine Biology*, 150(1), 149-160. https://doi.org/10.1007/s00227-006-0334-0

Güven, O., Gökdağ, K., Jovanović, B., Kıdeyş, A.E., 2017. Microplastic litter composition of the Turkish territorial waters of the Mediterranean Sea, and its occurrence in the gastrointestinal tract of fish. *Environmental Pollution* 223, 286-294. https://doi.org/10.1016/j.envpol.2017.01.025

Hernandez-Gonzalez, A., Saavedra, C., Gago, J., Covelo, P., Santos, M. B., & Pierce, G. J., 2018. Microplastics in the stomach contents of common dolphin (*Delphinus delphis*) stranded on the Galician coasts (NW Spain, 2005–2010). *Marine Pollution Bulletin*, 137, 526-532. https://doi.org/10.1016/j.marpolbul.2018.10.026

Herzke, D., Anker-Nilssen, T., Nøst, T.H., Götsch, A., Christensen-Dalsgaard, S., Langset, M., Fangel, K., Koelmans, A.A., 2016. Negligible impact of ingested microplastics on tissue concentrations of persistent organic pollutants in northern fulmars off coastal Norway. *Environmental Science & Technology* 50(4), 1924-1933. https://www.doi.org/10.1021/acs.est.5b04663

Hulley, P., 2015. *Lampanyctus crocodilus*. The IUCN Red List of Threatened Species 2015: e.T198618A15584685. https://dx.doi.org/10.2305/IUCN.UK.2015-4.RLTS.T198618A15584685.en. Downloaded on 07 October 2021.

Jamieson, A.J., Brooks, L.S.R., Reid, W.D.K., Piertney, S.B., Narayanaswamy, B.E., Linley, T.D., 2019. Microplastics and synthetic particles ingested by Deep-sea amphipods in six of the deepest marine ecosystems on Earth. *Royal Society Open Science* 6(2), 180667. https://doi.org/10.1098/rsos.180667

Jung, J., Park, J., Eo, S., Choi, J., Song, Y.K., Cho, Y., Hong, S.H., Shim, W.J., 2021. Ecological risk assessment of microplastics in coastal, shelf, and deep sea waters with a consideration of environmentally relevant size and shape. *Environmental Pollution* 270, 116217. https://doi.org/10.1016/j.envpol.2020.116217

Kaandrop, M.L.A., Dijkstra, H.A., van Sebille, E., 2020. Closing the Mediterranean marine floatinc plastic mass budget: inverse modeling of sources and sinks. *Environmental Science and Technology* 54(19), 11980 – 11989. https://doi.org/10.1021/acs.est.0c01984

Kaiser, D., Kowalski, N., Waniek, J.J., 2017. Effects of biofouling on the sinking behavior of microplastics. *Environmental Research Letters* 12, 124003. https://doi.org/10.1088/1748-9326/aa8e8b

Kazour, M., Jemaa, S., Issa, C., Khalaf, G., Amara, R., 2019. Microplastics pollution along the Lebanese coast (Eastern Mediterranean Basin): Occurrence in surface water, sediments and biota samples. *Science of the Total Environment* 696, 133933. https://doi.org/10.1016/j.scitotenv.2019.133933

Koelmans, A. A., Bakir, A., Burton, G. A., Janssen, C.R., 2016. Microplastic as a vector for chemicals in the aquatic environment: critical review and model-supported reinterpretation of empirical studies. *Environmental Science & Technology* 50(7), 3315-3326. https://doi.org/10.1021/acs.est.5b06069

Lohmann, R., 2017. Microplastics are not important for the cycling and bioaccumulation of organic pollutants in the oceans—but should microplastics be considered POPs themselves? *Integrated Environmental Assessment and Management* 13(3), 460-465. https://doi.org/10.1002/ieam.1914

Lusher, A. L., Mchugh, M., Thompson, R. C., 2013. Occurrence of microplastics in the gastrointestinal tract of pelagic and demersal fish from the English Channel. *Marine Pollution Bulletin* 67(1-2), 94-99. https://doi.org/10.1016/j.marpolbul.2012.11.028

Lusher, A. L., Burke, A., O'Connor, I., Officer, R., 2014. Microplastic pollution in the Northeast Atlantic Ocean: validated and opportunistic sampling. *Marine Pollution Bulletin* 88(1-2), 325-333. https://doi.org/10.1016/j.marpolbul.2014.08.023

Lusher, A.L., O'Donnell, C., Officer, R., O'Connor, I., 2016. Microplastic interactions with North Atlantic mesopelagic fish. *ICES Journal of Marine Science* 73, 1214–1225. https://doi.org/10.1093/icesjms/fsv241

Macias, D., Cózar, A., Garcia-Gorriz, E., González-Fernández, D., Stips, A., 2019. Surface water circulation develops seasonally changing patterns of floating litter accumulation in the Mediterranean Sea. A modelling approach. *Marine Pollution Bulletin* 149, 110619. https://doi.org/10.1016/j.marpolbul.2019.110619

Mateu, P., Nardi, V., Fraija-Fernández, N., Mattiucci, S., de Sola, L.G., Raga, J.A., Fernández, M., Aznar, F.J., 2015. The role of lantern fish (Myctophidae) in the life-cycle of cetacean parasites from western Mediterranean waters. *Deep Sea Research Part I: Oceanographic Research Papers* 95, 115 – 121. https://doi.org/10.1016/j.dsr.2014.10.012

Montarsolo, A., Mossotti, R., Patrucco, A., Caringella, R., Zoccola, M., Pozzo, P. D., Tonin, C., 2018. Study on the microplastics release from fishing nets. *The European Physical Journal Plus*, 133(11), 494. https://doi.org/10.1140/epjp/i2018-12415-1

Müller, C., Erzini, K., Teodósio, M. A., Pousão-Ferreira, P., Baptista, V., Ekau, W., 2020. Assessing microplastic uptake and impact on omnivorous juvenile white seabream *Diplodus sargus* (Linnaeus, 1758) under laboratory conditions. *Marine Pollution Bulletin* 157, 111162. https://doi.org/10.1016/j.marpolbul.2020.111162

Nelms, S.E., Galloway, T.S., Godley, B.J., Jarvis, D.S., Lindeque, P.K., 2018. Investigating microplastic trophic transfer in marine top predators. *Environmental Pollution* 238, 999–1007. https://doi.org/10.1016/j.envpol.2018.02.016

Neves, D., Sobral, P., Ferreira, J. L., Pereira, T., 2015. Ingestion of microplastics by commercial fish off the Portuguese coast. *Marine Pollution Bulletin*, 101(1), 119-126. https://doi.org/10.1016/j.marpolbul.2015.11.008

Novillo O., Raga, J.A., Tomás, J., 2020. Evaluating the presence of microplastics in striped dolphins (*Stenella coeruleoalba*) stranded in the Western Mediterranean Sea. *Marine Pollution Bulletin* 160, 111557. https://doi.org/10.1016/j.marpolbul.2020.111557

Novillo-Sanjuan, O., Raga, J.A., Tomás, J., 2022. Microdebris in three Spanish Mediterranean beaches located at a sporadic loggerhead turtles' (*Caretta caretta*) nesting area. *Regional Studies in Marine Science* 49, 102116. https://doi.org/10.1016/j.rsma.2021.102116

Olivar, M.P., Bernal, A., Molí, M., Balbín, R., Castellón, A., Miquel, J., Massutí, E., 2012. Vertical distribution, diversity and assemblages of mesopelagic fishes in the western Mediterranean. *Deep Sea Research Part I: Oceanographic Research Papers* 62, 53 – 69. https://doi.org/10.1016/j.dsr.2011.12.014

Pham, C.K., Ramirez-Llodra, E., Alt, C.H.S., Amaro, T., Bergmann, M., Canals, M., Company, J.B., Davies, J., Duineveld, G., Galgani, F., Howell, K.L., Huvenne, V.A.I., Isidro, E., Jones, D.O.B., Lastras, G., Morato, T., Gomes-Pereira, J.N., Purser, A., Stewart, H., Tojeira, I., Tubau, X., Van Rooij, D., Tyler, P.A., 2014. Marine Litter Distribution and Density in European Seas, from the Shelves to Deep Basins. *PLoS One* 9, e95839. https://doi.org/10.1371/journal.pone.0095839

R Core Team, 2019. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/

Reiczigel, J., Marozzi, M., Fabian, I., Rozsa, L., 2019. Biostatistics for parasitologists – a primer to Quantitative Parasitology, *Trends in Parasitology* 35(4): 277-281. https://www.doi.org/10.1016/j.pt.2019.01.003

Reissner, J., Slat, B., Noble, K., du Plessis, K., Epp, M., Proietti, M., de Sonneville, J., Becker, T., Pattiaratchi, C., 2015. The vertical distribution of buoyant plastics at sea: an observational study in the North Atlantic Gyre. *Biogeosciences* 12, 1249 – 1256. https://doi.org/10.5194/bg-12-1249-2015

Rios-Fuster, B., Alomar, C., Compa, M., Guijarro, B., Deudero, S., 2019. Anthropogenic particles ingestion in fish species from two areas of the western Mediterranean Sea. *Marine Pollution Bulletin* 144, 325-333. https://doi.org/10.1016/j.marpolbul.2019.04.064

Roch, S., Friedrich, C., Brinker, A., 2020. Uptake routes of microplastics in fishes: practical and theoretical approaches to test existing theories. *Science Reports* 10, 3896. https://doi.org/10.1038/s41598-020-60630-1

Rochman, C.M., Lewison, R.L., Eriksen, M., Allen, H., Cook, A.-M., Teh, S.J., 2014. Polybrominated diphenyl ethers (PBDEs) in fish tissue may be an indicator of plastic contamination in marine habitats. *Science of The Total Environment* 476–477, 622–633. https://doi.org/10.1016/j.scitotenv.2014.01.058

Rodrigues, J.P., Duarte, A.C., Santos-Echeandía, J., Rocha-Santos, T., 2019. Significance of interactions between microplastics and POPs in the marine environment: a critical overview. *TrAC Trends in Analytical Chemistry* 111, 252 – 260. https://doi.org/10.1016/j.trac.2018.11.038

Roman, J., McCarthy, J. J., 2010. The whale pump: marine mammals enhance primary productivity in a coastal basin. *PloS one*, 5(10), e13255. https://doi.org/10.1371/journal.pone.0013255

Roman, J., Estes, J. A., Morissette, L., Smith, C., Costa, D., McCarthy, J., ... Smetacek, V., 2014. Whales as marine ecosystem engineers. *Frontiers in Ecology and the Environment*, 12(7), 377-385. https://doi.org/10.1890/130220

Romeo, T., Pedà, C., Fossi, M.C., Andaloro, F., Battaglia, P., 2016. First record of plastic debris in the stomach of Mediterranean lanternfishes. *Acta Adriatica* 57(1), 113 – 122. ISSN: 0001-5113

Ryan, M. G., Watkins, L., Walter, M. T., 2019. Hudson River juvenile Blueback herring avoid ingesting microplastics. *Marine Pollution Bulletin* 146, 935-939. https://doi.org/10.1016/j.marpolbul.2019.07.004

Saavedra, C., García-Polo, M., Giménez, J., Mons, J. L., Castillo, J. J., Fernández-Maldonado, C., Stephanis, R., Pierce, G.J., Santos, M. B., 2022. Diet of striped dolphins (*Stenella coeruleoalba*) in southern Spanish waters. *Marine Mammal Science*.https://doi.org/10.1111/mms.12945

Savoca, S., Capillo, G., Mancuso, M., Bottari, T., Crupi, R., Branca, C., ... Spanò, N., 2019. Microplastics occurrence in the Tyrrhenian waters and in the gastrointestinal tract of two congener species of seabreams. *Environmental toxicology and pharmacology*, 67, 35-41. https://doi.org/10.1016/j.etap.2019.01.011

Savoca, M. S., Czapanskiy, M. F., Kahane-Rapport, S. R., Gough, W. T., Fahlbusch, J. A., Bierlich, K. C., ... & Goldbogen, J. A., 2021. Baleen whale prey consumption based on high-resolution foraging measurements. *Nature*, 599(7883), 85-90. https://doi.org/10.1038/s41586-021-03991-5

Sayed, A. E. D. H., Hamed, M., Badrey, A. E., Ismail, R. F., Osman, Y. A., Osman, A. G., Soliman, H. A., 2021. Microplastic distribution, abundance, and composition in the sediments, water, and fishes of the Red and Mediterranean seas, Egypt. *Marine Pollution Bulletin*, 173, 112966. https://doi.org/10.1016/j.marpolbul.2021.112966

Setälä, O., Fleming-Lehtinen, V., Lehtiniemi, M., 2014. Ingestion and transfer of microplastics in the planktonic food web. *Environmental Pollution* 185, 77 – 83. https://doi.org/10.1016/j.envpol.2013.10.013

Silva, A. B., Bastos, A. S., Justino, C. I., da Costa, J. P., Duarte, A. C., & Rocha-Santos, T. A., 2018. Microplastics in the environment: Challenges in analytical chemistry-A review. *Analytica Chimica Acta*, 1017, 1-19. https://doi.org/10.1016/j.aca.2018.02.043

Stefanescu, C. & Cartes, J.E., 1992. Benthopelagic habits of adult specimens of *Lampanyctus crocodilus* (Risso, 1810) (Osteichthyes, Myctophidae) in the western Mediterranean deep slope. *Scientia Marina* 56, 69 – 74. ISSN: 0214-8358

Teuten, E.L., Saquing, J.M., Knappe, D.R.U., Barlaz, M.A., Jonsson, S., Björn, A., Rowland, S.J., Thompson, R.C., Galloway, T.S., Yamashita, R., Ochi, D., Watanuki, Y., Moore, C., Viet, P.H., Tana, T.S., Prudente, M., Boonyatumanond, R., Zakaria, M.P., Akkhavong, K., Ogata, Y., Hirai, H., Iwasa, S., Mizukawa, K., Hagino, Y., Imamura, A., Saha, M., Takada, H., 2009. Transport and release of chemicals from plastics to the environment and to wildlife. *Philosophical Transactions of the Royal Society B* 364, 2027–2045. https://doi.org/10.1098/rstb.2008.0284

Valls, M., Olivar, M.P., Fernández de Puelles, M.L., Molí, B., Bernal, A., Sweeting, C.J., 2014. Trophic structure of mesopelagic fishes in the western Mediterranean based on stable isotopes of carbon and nitrogen. Journal of Marine Systems, The wrapping up of the IDEADOS project: International Workshop on Environment, Ecosystems and Demersal Resources, and Fisheries 138, 160–170. https://doi.org/10.1016/j.jmarsys.2014.04.007

van Sebille, E., Wilcox, C., Lebreton, L., Maximenko, N., Hardesty, B.D., van Franeker, J.A., Eriksen, M., Siegel, D., Galgani, F., Law, K.L., 2015. A global inventory of small floating plastic debris. *Environmental Research Letters* 10, 124006. https://doi.org/10.1088/1748-9326/10/12/124006

Wang, Z.M., Wagner, J., Ghosal, S., Bedi, G., Wall, S., 2017. SEM/EDS and optical microscopy analyses of microplastics in ocean trawl and fish guts. *Science of the Total Environment* 15, 616 – 626. https://doi.org/10.1016/j.scitotenv.2017.06.047

Wang, W.H., Gao, H., Jin, S., Li, R., Na, G., 2019. The ecotoxicological effects of microplastics on aquatic food web, from primary producer to human: a review. *Ecotoxicology and Environmental Safety* 173, 110 – 117. https://doi.org/10.1016/j.ecoenv.2019.01.113

Ward, J.E., Zhao, S., Holohan, B.A., Mladinich, K.M., Griffin, T.W., Wozniak, J., Shumway, S.E., 2019. Selective Ingestion and Egestion of Plastic Particles by the Blue Mussel (*Mytilus edulis*) and Eastern Oyster (Crassostrea virginica): Implications for Using Bivalves as Bioindicators of Microplastic Pollution. *Environmental Science Technology* 53, 8776–8784. https://doi.org/10.1021/acs.est.9b02073

Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.

Wieczorek, A. M., Morrison, L., Croot, P. L., Allcock, A. L., MacLoughlin, E., Savard, O., Brownlow, H., Doyle, T. K. 2018. Frequency of microplastics in mesopelagic fishes from the Northwest Atlantic. *Frontiers in Marine Science*, 39. https://doi.org/10.3389/fmars.2018.00039

Woodall, L. C., Sanchez-Vidal, A., Canals, M., Paterson, G. L., Coppock, R., Sleight, V., Calafat, A., Narayanaswamy, B.E., Thompson, R. C., 2014. The deep sea is a major sink for microplastic debris. *Royal Society open science*, 1(4), 140317. https://doi.org/10.1098/rsos.140317

Zhang, H., 2017. Transport of microplastics in coastal areas. *Estuarine, coastal and shelf science* 199, 74 – 86. https://doi.org/10.1016/j.ecss.2017.09.032

GENERAL CONCLUSIONS

Pollutants leached into the environment, not only remain in abiotic matrices, they are also incorporated into biota through different pathways. Studies in wild, non-commercial species are not as abundant but are equally important to identify and quantify pollutants at sea and determine thresholds to reach a good environmental status in the Mediterranean Sea. In this thesis, we highlight the presence of different pollutants of concern in understudied, non-commercial species, including the loggerhead sea turtle (and their potential nesting habitats), the striped dolphin and the jewel latern fish collected in waters of the central Spain's Mediterranean. The studies carried out yielded the following conclusions:

1. The non-targeted analyses showed up to 39 different chemicals in the studied stock of loggerhead sea turtles from the western Mediterranean, 38.5% of these chemicals were banned in the EU. The non-approved pesticides could have been incorporated while the turtles were diving and foraging through non-EU Mediterranean waters in which those are not banned, or in Mediterranean EU-waters where, although forbidden, they are still illegaly used. Except the DDT detected in one sample, the rest of pesticides can be quickly metabolized by the organism, hence their presence indicates continuous exposure. Unfortunately, the lack of toxicity thresholds for marine turtles makes it difficult to assess potential effects of these pesticides on turtles' health.

2. Metal content in loggerhead turtles from this study was generally low and were not correlated with size (measured as CCL), nor with the geographic area where samples were collected. Mean concentrations of Cd, Pb and Hg were 0.04 µg/g w.w., 0.09 µg/g w.w. and 0.03 µg/g w.w. in fat and 0.05 µg/g w.w., 0.08 µg/g w.w. and 0.04 µg/g w.w. in muscle, respectively. Loggerhead turtles in this study have lower levels of metals in their tissues than in other studies from the eastern Mediterranean, probably due to the proximity to the Gibraltar Strait, where there is water exchange with the Atlantic Ocean and, therefore, pollution in this part of the Mediterranean can be diluted or moved to the East pushed by sea currents from the Atlantic. Hence, metal pollution does not seem to be an important threat to western Mediterranean loggerhead turtles.

3. Loggerhead turtles are usually in touch with plastic additives, which confirms the ubiquity of these pollutants in the environment and the potential interaction of marine fauna with plastics and other products where those are included. Phthalate metabolites showed, in general, a high DR. Seven out of 18 metabolites analysed showed DR≥85% (mMP, PA, mEP, mBP, mHxP, mNP, and mHepP). The metabolite with a highest mean was mDEP (38.9 ng/g d.w.), followed by mEHP (22.2 ng/g d.w.), and mHxP (20.2 ng/g d.w.). In this case, metabolites concentrations showed a steep increase from 2020 onwards, indicating either a possible increase in single-use plastic consumption and disposal following the COVID-19 pandemic, or an increase in uncontrolled waste water discharges into the coastal areas.

4. Microdebris concentration in the three potential sea turtle nesting beaches studied in the Valencia province was low in comparison to other locations in the Mediterranean and elsewhere, although it was always present. Total mean±SD in July was 5.66 ± 3.66 MPs/kg at surface and 12.15 ± 7.76 MPs/kg at depth; while in November values were 6.45 ± 4.42 MPs/kg at surface and 5.51 ± 3.14 MPs/kg at depth. The spectrometric analysis revealed that most of the microplastics came from consumer goods, tires and food packaging. Polymers found were, by descent order, polyethylene, rubber, latex, polypropylene and ethylene vinyl alcohol.

5. Beach location, month, depth of sampling or protection regime of the beach did not account for differences in microdebris amount, meaning that the beaches under the natural park regime receive the same amount of litter than the urban beach, in spite of being further away from the city. This may be a consequence of sources being diffuse and widespread. Also, due to its low weight and density, microdebris is easily transported by air and water currents, hence, it is distributed along the coast.

6. Since these beaches can be potentially used by loggerhead turtles currently nesting in Spain's Mediterranean coasts, and since beach temperature is crucial for the success of clutch incubation, it is necessary to determine potential effects of microplastic concentration in sand samples. In this case, it seems very improbable that microdebris concentration could modify incubation temperature and, therefore, affect the sex ratio or the survival of loggerhead turtle hatchlings.

7. The striped dolphin is frequently exposed to microplastics, mostly fibers (73.6%), in the western Mediterranean. However, microplastic burden in their bodies is relatively low

for their body size and, apparently, does not pose a threat to their health. According to FT-IR, 40.9% of microplastics found were made of polyacrylamide, followed by PET and HDPE. Whether striped dolphins ingest microplastics directly from the surrounding water or through diet is still yet to be known. From this study we can also conclude that the use of striped dolphins as bioindicators of microplastic presence in pelagic biota is limited. The difficulty of sampling together with the very time-consuming process of analysing big digestive tracts, make this species less suitable for monitoring environmental health than others.

8. It was relatively frequent to find microplastics in the jewel lanternfish, since 40.21% of the sampled specimens had at least one of them. However, body burden was low, as most of the individuals had only one item in their gastrointestinal tracts (median was 3 MPs/fish, CI 95% = 3.46 – 6.8). In fact, microplastic content did not correlate with fish body condition nor their length. Since lanternfish constitute an important part of the oceanic biomass and are key species in the ecosystem and in the biological pump, they can provide valuable information about pollution presence and circulation in the bathypelagic environment. Furthermore, they are relatively easy to process, making them an interesting species to further study pollutants in the ocean.

9. The jewel lantern fish has been described as important prey of the striped dolphin, among other species. Here, we found microplastic items in both species; therefore, their potential role as vectors of this contaminant to predators in the marine food web could be further explored. However, further polymer analyses and studies about the ingestion and egestion rate of microplastics should be performed to be able to confirm that striped dolphins could be acquiring these particles via the myctophids they consume.

To conclude, in this thesis we have shown that pollution is present in all the analysed species and that it is already part of the normal marine environment. Pollutants are incorporated by marine organism, thus entering the marine food web. However, a deeper knowledge about toxicity thresholds in these species is needed to assess how these pollutants could affect species health and survival. Moreover, mitigation and protection plans are needed at the pollution sources to prevent chemical contaminants, microplastics and waste from reaching the sea wherever possible.

SCIENTIFIC ARTICLES RESULTTING FROM THIS THESIS

- Novillo, O., Pertusa, J. F., & Tomás, J., 2017. Exploring the presence of pollutants at sea: monitoring heavy metals and pesticides in loggerhead turtles (*Caretta caretta*) from the western Mediterranean. *Science of the Total Environment*, 598, 1130-1139. https://doi.org/10.1016/j.scitotenv.2017.04.090

- Novillo, O., Raga, J. A., & Tomás, J., 2020. Evaluating the presence of microplastics in striped dolphins (*Stenella coeruleoalba*) stranded in the Western Mediterranean Sea. *Marine Pollution Bulletin*, 160, 111557. https://doi.org/10.1016/j.marpolbul.2020.111557

- Novillo-Sanjuan, O., Raga, J. A., & Tomás, J., 2022. Microdebris in three Spanish Mediterranean beaches located at a sporadic loggerhead turtles' (*Caretta caretta*) nesting area. *Regional Studies in Marine Science*, 49, 102116. https://doi.org/10.1016/j.rsma.2021.102116

- Novillo-Sanjuan, O., Gallén, S., Raga, J. A., & Tomás, J., 2023 (Under review). Microplastics in *Lampanyctus crocodilus* (Myctophidae), a common lanternfish species from the Ibiza Channel (western Mediterranean). *Animals*.

- Novillo-Sanjuan, O., Sait, S. T., González, S. V., Raga, J. A., Tomás, J., Asimakopoulos, A. G., 2023. Phthalate metabolites in loggerhead marine turtles (*Caretta caretta*) from the Mediterranean Sea (East Spain region). In preparation.

GENERAL REFERENCES

Abalo-Morla, S., Marco, A., Tomás, J., Revuelta, O., Abella, E., Marco, V., ... & Belda, E. J., 2018. Survival and dispersal routes of head-started loggerhead sea turtle (*Caretta caretta*) post-hatchlings in the Mediterranean Sea. *Marine Biology*, *165*(3), 1-17. https://doi.org/10.1007/s00227-018-3306-2

Ackerman, R.A., 1997. The nest environment and the embryonic development of sea turtles. In: Lutz PL, Musick JA (Eds.) The biology of Sea Turtles (pp. 83-106). CRC Press, Boca Raton, FL.

Aguilar, A. & Gaspari, S., 2012. *Stenella coeruleoalba*. *The IUCN Red List of Threatened Species* 2012: e.T20731A2773889. Accessed on 24 August 2022.

Alava, J. J., 2020. Modeling the bioaccumulation and biomagnification potential of microplastics in a cetacean foodweb of the northeastern pacific: a prospective tool to assess the risk exposure to plastic particles. *Frontiers in Marine Science*, *7*, 566101. https://doi.org/10.3389/fmars.2020.566101

Alkan, N., Alkan, A., Castro-Jiménez, J., Royer, F., Papillon, L., Ourgaud, M., & Sempere, R., 2021. Environmental occurrence of phthalate and organophosphate esters in sediments across the Gulf of Lion (NW Mediterranean Sea). *Science of the total Environment*, *760*, 143412. https://doi.org/10.1016/j.scitotenv.2020.143412

Andrady, A. L., 2017. The plastic in microplastics: A review. *Marine pollution bulletin*, *119*(1), 12-22. https://doi.org/10.1016/j.marpolbul.2017.01.082

Asimakopoulos, A. G., Xue, J., De Carvalho, B. P., Iyer, A., Abualnaja, K. O., Yaghmoor, S. S., ... & Kannan, K., 2016. Urinary biomarkers of exposure to 57 xenobiotics and its association with oxidative stress in a population in Jeddah, Saudi Arabia. *Environmental research*, *150*, 573-581. https://doi.org/10.1016/j.envres.2015.11.029

Avio, C. G., Gorbi, S., & Regoli, F., 2017. Plastics and microplastics in the oceans: from emerging pollutants to emerged threat. *Marine environmental research*, *128*, 2-11. https://doi.org/10.1016/j.envpol.2017.04.066

Aznar, F. J., Míguez-Lozano, R., Ruiz, B., de Castro, A. B., Raga, J. A., & Blanco, C., 2017. Long-term changes (1990-2012) in the diet of striped dolphins *Stenella coeruleoalba* from the western Mediterranean. *Marine Ecology Progress Series*, *568*, 231-247. https://doi.org/10.3354/meps12063

Beckwith, V. K., & Fuentes, M. M., 2018. Microplastic at nesting grounds used by the northern Gulf of Mexico loggerhead recovery unit. *Marine pollution bulletin*, *131*, 32-37. https://doi.org/10.1016/j.marpolbul.2018.04.001

Besley, A., Vijver, M. G., Behrens, P., & Bosker, T., 2017. A standardized method for sampling and extraction methods for quantifying microplastics in beach sand. Marine Pollution Bulletin, 114(1), 77-83. https://doi.org/10.1016/j.marpolbul.2016.08.055

Bianchi, C. N., & Morri, C., 2000. Marine biodiversity of the Mediterranean Sea: situation, problems and prospects for future research. *Marine pollution bulletin*, *40*(5), 367-376. https://doi.org/10.1016/S0025-326X(00)00027-8

Blanco, C., Aznar, J., & Raga, J. A., 1995. Cephalopods in the diet of the striped dolphin *Stenella coeruleoalba* from the western Mediterranean during an epizootic in 1990. *Journal of Zoology*, *237*(1), 151-158. https://doi.org/10.1111/j.1469-7998.1995.tb02753.x

Bolten, A. B., & Witherington, B. E., 2004. Loggerhead sea turtles. *Marine Turtle Newsl*, *104*, 319.

Bonanno, G., & Orlando-Bonaca, M., 2018. Perspectives on using marine species as bioindicators of plastic pollution. *Marine pollution bulletin*, *137*, 209-221. https://doi.org/10.1016/j.marpolbul.2018.10.018

Booth, D. T., 2017. Influence of incubation temperature on sea turtle hatchling quality. *Integrative Zoology*, *12*(5), 352-360. https://doi.org/10.1111/1749-4877.12255

Borrelle, S. B., Ringma, J., Law, K. L., Monnahan, C. C., Lebreton, L., McGivern, A., ... & Rochman, C. M., 2020. Predicted growth in plastic waste exceeds efforts to mitigate plastic pollution. *Science*, *369*(6510), 1515-1518. https://doi.org/10.1126/science.aba365

Bowman, D., Manor-Samsonov, N., & Golik, A., 1998. Dynamics of litter pollution on Israeli Mediterranean beaches: a budgetary, litter flux approach. *Journal of Coastal Research*, 418-432. ISSN: 079-0208.

Brasfield, S. M., Bradham, K., Wells, J. B., Talent, L. G., Lanno, R. P., & Janz, D. M., 2004. Development of a terrestrial vertebrate model for assessing bioavailability of cadmium in the fence lizard (*Sceloporus undulatus*) and in ovo effects on hatchling size and thyroid function. *Chemosphere*, *54*(11), 1643-1651. https://doi.org/10.1016/j.chemosphere.2003.09.030

Braulik, G., 2019. *Stenella coeruleoalba. The IUCN Red List of Threatened Species* 2019: e.T20731A50374282. https://dx.doi.org/10.2305/IUCN.UK.2019-1.RLTS.T20731A50374282.en. Accessed on 24 August 2022.

Bucchia, M., Camacho, M., Santos, M. R., Boada, L. D., Roncada, P., Mateo, R., ... & Luzardo, O. P., 2015. Plasma levels of pollutants are much higher in loggerhead turtle populations from the Adriatic Sea than in those from open waters (Eastern Atlantic Ocean). *Science of the Total Environment*, *523*, 161-169. https://doi.org/10.1016/j.scitotenv.2015.03.047

Burger, J., & Gibbons, J. W., 1998. Trace elements in egg contents and egg shells of slider turtles (*Trachemys scripta*) from the Savannah River Site. *Archives of environmental contamination and toxicology*, *34*(4), 382-386. https://doi.org/10.1007/s002449900334

Burger, J., 2008. Assessment and management of risk to wildlife from cadmium. *Science of the total environment*, *389*(1), 37-45. https://doi.org/10.1016/j.scitotenv.2007.08.037

Campo, J., Masiá, A., Blasco, C., & Picó, Y., 2013. Occurrence and removal efficiency of pesticides in sewage treatment plants of four Mediterranean River Basins. *Journal of hazardous materials*, *263*, 146-157. https://doi.org/10.1016/j.jhazmat.2013.09.061

Campos, É., & Freire, C., 2016. Exposure to non-persistent pesticides and thyroid function: A systematic review of epidemiological evidence. *International journal of hygiene and environmental health*, *219*(6), 481-497. https://doi.org/10.1016/j.ijheh.2016.05.006

Carreras, C., Pascual, M., Tomás, J., Marco, A., Hochscheid, S., Castillo, J. J., ... & Cardona, L., 2018. Sporadic nesting reveals long distance colonisation in the philopatric loggerhead sea turtle (*Caretta caretta*). *Scientific reports*, *8*(1), 1-14. https://doi.org/10.1038/s41598-018-19887-w

Cardona, L., Álvarez de Quevedo, I., Borrell, A., & Aguilar, A., 2012. Massive consumption of gelatinous plankton by Mediterranean apex predators. *PloS one*, *7*(3), e31329.

Carson, H. S., Colbert, S. L., Kaylor, M. J., & McDermid, K. J., 2011. Small plastic debris changes water movement and heat transfer through beach sediments. *Marine Pollution Bulletin*, *62*(8), 1708-1713. https://doi.org/10.1016/j.marpolbul.2011.05.032

Casale, P., & Tucker, A. D., 2015. *Caretta caretta*. The IUCN Red List of Threatened Species 2015: e. T3897A83157651.

Casale, P., & Heppell, S. S., 2016. How much sea turtle bycatch is too much? A stationary age distribution model for simulating population abundance and potential biological removal in the Mediterranean. *Endangered Species Research*, *29*(3), 239-254. https://doi.org/10.3354/esr00714

Ccanccapa, A., Masiá, A., Andreu, V., & Picó, Y., 2016a. Spatio-temporal patterns of pesticide residues in the Turia and Júcar Rivers (Spain). *Science of the Total Environment*, *540*, 200-210. https://doi.org/10.1016/j.scitotenv.2015.06.063

Ccanccapa, A., Masiá, A., Navarro-Ortega, A., Picó, Y., & Barceló, D., 2016b. Pesticides in the Ebro River basin: occurrence and risk assessment. *Environmental Pollution*, *211*, 414-424. https://doi.org/10.1016/j.envpol.2015.12.059

Ceriani, S. A., Roth, J. D., Evans, D. R., Weishampel, J. F., & Ehrhart, L. M., 2012. Inferring foraging areas of nesting loggerhead turtles using satellite telemetry and stable isotopes. https://doi.org/10.1371/journal.pone.0045335

Cinnirella, S., Graziano, M., Pon, J., Murciano, C., Albaigés, J., & Pirrone, N., 2013. Integrated assessment of chemical pollution in the Mediterranean Sea: Driver-Pressures-State-Welfare analysis. *Ocean & coastal management*, *80*, 36-45. https://doi.org/10.1016/j.ocecoaman.2013.02.022

Cinnirella, S., Bruno, D. E., Pirrone, N., Horvat, M., Živković, I., Evers, D. C., ... & Sunderland, E. M., 2019. Mercury concentrations in biota in the Mediterranean Sea, a compilation of 40 years of surveys. *Scientific data*, *6*(1), 1-11. https://doi.org/10.6084/m9.figshare.9886004

Cole, M., Lindeque, P., Halsband, C., & Galloway, T. S., 2011. Microplastics as contaminants in the marine environment: a review. *Marine pollution bulletin*, *62*(12), 2588-2597. https://doi.org/10.1016/j.marpolbul.2011.09.025

Compa, M., Alomar, C., Wilcox, C., van Sebille, E., Lebreton, L., Hardesty, B. D., & Deudero, S., 2019. Risk assessment of plastic pollution on marine diversity in the Mediterranean Sea. *Science of The Total Environment*, *678*, 188-196. https://doi.org/10.1016/j.scitotenv.2019.04.355

Constant, M., Kerhervé, P., Mino-Vercellio-Verollet, M., Dumontier, M., Vidal, A. S., Canals, M., & Heussner, S., 2019. Beached microplastics in the northwestern Mediterranean Sea. *Marine pollution bulletin*, *142*, 263-273. https://doi.org/10.1016/j.marpolbul.2019.03.032

Cózar, A., Sanz-Martín, M., Martí, E., González-Gordillo, J. I., Ubeda, B., Gálvez, J. Á., ... & Duarte, C. M., 2015. Plastic accumulation in the Mediterranean Sea. *PloS one*, *10*(4), e0121762. https://doi.org/10.1371/journal.pone.0121762

Darmon, G., Schulz, M., Matiddi, M., Loza, A. L., Tòmàs, J., Camedda, A., ... & Miaud, C., 2022. Drivers of litter ingestion by sea turtles: Three decades of empirical data collected in Atlantic Europe and the Mediterranean. Marine Pollution Bulletin, 185, 114364. https://doi.org/10.1016/j.marpolbul.2022.114364

Davison, P., & Asch, R. G., 2011. Plastic ingestion by mesopelagic fishes in the North Pacific Subtropical Gyre. *Marine Ecology Progress Series*, *432*, 173-180. https://doi.org/10.3354/meps09142

De Frond, H. L., van Sebille, E., Parnis, J. M., Diamond, M. L., Mallos, N., Kingsbury, T., & Rochman, C. M., 2019. Estimating the mass of chemicals associated with ocean plastic pollution to inform mitigation efforts. *Integrated environmental assessment and management*, *15*(4), 596-606. https://doi.org/10.1002/ieam.4147

Di Lorenzo, M., Barra, T., Rosati, L., Valiante, S., Capaldo, A., De Falco, M., & Laforgia, V., 2020. Adrenal gland response to endocrine disrupting chemicals in fishes, amphibians and reptiles: A comparative overview. *General and comparative endocrinology*, *297*, 113550. https://doi.org/10.1016/j.ygcen.2020.113550

Diamond, M. L., de Wit, C. A., Molander, S., Scheringer, M., Backhaus, T., Lohmann, R., ... & Zetzsch, C., 2015. Exploring the planetary boundary for chemical pollution. *Environment international*, *78*, 8-15. https://doi.org/10.1016/j.envint.2015.02.001

Encarnação, T., Pais, A. A., Campos, M. G., & Burrows, H. D., 2019. Endocrine disrupting chemicals: Impact on human health, wildlife and the environment. *Science progress*, *102*(1), 3-42. https://doi.org/10.1177/0036850419826802

Domènech, F., Aznar, F. J., Raga, J. A., & Tomás, J., 2019. Two decades of monitoring in marine debris ingestion in loggerhead sea turtle, *Caretta caretta*, from the western Mediterranean. *Environmental Pollution*, *244*, 367-378. https://doi.org/10.1016/j.envpol.2018.10.047

European Chemical Agency. August, 2022. *Microplastics*. ECHA. https://echa.europa.eu/hot-topics/microplastics

Eder, E., Ceballos, A., Martins, S., Pérez-García, H., Marín, I., Marco, A., & Cardona, L., 2012. Foraging dichotomy in loggerhead sea turtles *Caretta caretta* off northwestern Africa. *Marine Ecology Progress Series*, *470*, 113-122. https://doi.org/10.3354/meps10018

Eriksen, M., Lebreton, L. C., Carson, H. S., Thiel, M., Moore, C. J., Borerro, J. C., ... & Reisser, J., 2014. Plastic pollution in the world's oceans: more than 5 trillion plastic pieces weighing over 250,000 tons afloat at sea. *PloS one*, *9*(12), e111913. https://doi.org/10.1371/journal.pone.0111913

Esposito, M., De Roma, A., Sansone, D., Capozzo, D., Iaccarino, D., di Nocera, F., & Gallo, P., 2020. Non-essential toxic element (Cd, As, Hg and Pb) levels in muscle, liver and kidney of loggerhead sea turtles (*Caretta caretta*) stranded along the southwestern coasts of Tyrrhenian sea. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *231*, 108725. https://doi.org/10.1016/j.cbpc.2020.108725

Reker, J., Murray, C., Royo-Gelabert, E., Abhold, K., Korpinen, S., Peterlin, M., Vaughan, D., & Andersen, J.H., 2019. *Marine messages II: Navigating the course towards clean, healthy and productive seas* *through implementation of an ecosystem-based approach* (EEA Report No 17/2019). European Environment Agency. https://doi.org/10.2800/71245

Faccini, F., Luino, F., Paliaga, G., Roccati, A., & Turconi, L., 2021. Flash flood events along the west mediterranean coasts: Inundations of urbanized areas conditioned by anthropic impacts. *Land*, *10*(6), 620. https://doi.org/10.3390/land10060620

Fanelli, E., Papiol, V., Cartes, J. E., & Rodriguez-Romeu, O., 2014. Trophic ecology of Lampanyctus crocodilus on north-west Mediterranean Sea slopes in relation to reproductive cycle and environmental variables. *Journal of Fish Biology*, *84*(6), 1654-1688. https://doi.org/10.1111/jfb.12378

Febrer-Serra, M., Renga, E., Fernández, G., Lassnig, N., Tejada, S., Capó, X., ... & Sureda, A., 2020. First report of heavy metal presence in muscular tissue of loggerhead turtles Caretta caretta (Linnaeus, 1758) from the Balearic Sea (Balearic Islands, Spain). *Environmental Science and Pollution Research*, *27*(31), 39651-39656. https://doi.org/10.1007/s11356-020-10464-1

Fernández, S. F., Pardo, O., Corpas-Burgos, F., & Yusà, V., 2020. Exposure and cumulative risk assessment to non-persistent pesticides in Spanish children using biomonitoring. *Science of The Total Environment*, *746*, 140983. https://doi.org/10.1016/j.scitotenv.2020.140983

Finlayson, K. A., Leusch, F. D., & van de Merwe, J. P., 2016. The current state and future directions of marine turtle toxicology research. *Environment international*, *94*, 113-123. https://doi.org/10.1016/j.envint.2016.05.013

Fisher, L. R., Godfrey, M. H., & Owens, D. W., 2014. Incubation temperature effects on hatchling performance in the loggerhead sea turtle (*Caretta caretta*). *PLoS One*, *9*(12), e114880. https://doi.org/10.1371/journal.pone.0114880

Fossi, M. C., Romeo, T., Baini, M., Panti, C., Marsili, L., Campani, T., ... & Lapucci, C., 2017. Plastic debris occurrence, convergence areas and fin whales feeding ground in the Mediterranean marine protected area Pelagos sanctuary: a modeling approach. *Frontiers in marine science*, 167. https://doi.org/10.3389/fmars.2017.00167

Fossi, M. C., Pedà, C., Compa, M., Tsangaris, C., Alomar, C., Claro, F., ... & Baini, M., 2018. Bioindicators for monitoring marine litter ingestion and its impacts on Mediterranean biodiversity. *Environmental Pollution*, *237*, 1023-1040. https://doi.org/10.1016/j.envpol.2017.11.019

Fraija-Fernández, N., Gozalbes, P., Tomás, J., Balbuena, J.A., Domènech, F., Eymar, J., Mateu, P., Míguez, R., Revuelta, O., Raga, J.A., 2015. Long term boat-based surveys in the Central Spanish Mediterranean (2003-2013): Cetacean diversity and distribution. 29th Annual Conference of the European Cetacean Society. Malta.

Franzellitti, S., Locatelli, C., Gerosa, G., Vallini, C., & Fabbri, E., 2004. Heavy metals in tissues of loggerhead turtles (*Caretta caretta*) from the northwestern Adriatic Sea. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *138*(2), 187-194. https://doi.org/10.1016/j.cca.2004.07.008

Froese, R., & Pauly, D. (Eds.)., 2000. *FishBase 2000: concepts designs and data sources* (Vol. 1594). WorldFish. ISSN: 0116-6964.

Froese, R., Winker, H., Coro, G., Demirel, N., Tsikliras, A. C., Dimarchopoulou, D., ... & Matz-Lück, N., 2018. Status and rebuilding of European fisheries. *Marine Policy*, *93*, 159-170. https://doi.org/10.1016/j.marpol.2018.04.018

Freer, J. J., Tarling, G. A., Collins, M. A., Partridge, J. C., & Genner, M. J., 2019. Predicting future distributions of lanternfish, a significant ecological resource within the Southern Ocean. *Diversity and Distributions*, *25*(8), 1259-1272. https://doi.org/10.1111/ddi.12934

Froese, R., 2006. Cube law, condition factor and weight–length relationships: history, meta-analysis and recommendations. *Journal of Applied Ichthyology* 22(4), 241-253. https://doi.org/10.1111/j.1439-0426.2006.00805.x

Gago, J., Galgani, F., Maes, T., & Thompson, R. C., 2016. Microplastics in seawater: recommendations from the marine strategy framework directive implementation process. *Frontiers in Marine Science*, *3*, 219. https://doi.org/10.3389/fmars.2016.00219

Galloway, T. S., Cole, M., & Lewis, C., 2017. Interactions of microplastic debris throughout the marine ecosystem. *Nature ecology & evolution*, *1*(5), 1-8. https://doi.org/10.1038/s41559-017-0116

García-Fernández, A. J., Gómez-Ramírez, P., Martínez-López, E., Hernández-García, A., María-Mojica, P., Romero, D., ... & Bellido, J. J., 2009. Heavy metals in tissues from loggerhead turtles (*Caretta caretta*) from the southwestern Mediterranean (Spain). *Ecotoxicology and Environmental Safety*, *72*(2), 557-563. https://doi.org/10.1016/j.ecoenv.2008.05.003

GESAMP (2015). "Sources, fate and effects of microplastics in the marine environment: a global assessment" (Kershaw, P. J., ed.). (IMO/FAO/UNESCO-IOC/UNIDO/WMO/IAEA/UN/UNEP/UNDP Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection). Rep. Stud. GESAMP No. 90, 96 p.

Gómez, M. J., Bueno, M. M., Lacorte, S., Fernández-Alba, A. R., & Agüera, A., 2007. Pilot survey monitoring pharmaceuticals and related compounds in a sewage treatment plant located on the Mediterranean coast. *Chemosphere*, *66*(6), 993-1002. https://doi.org/10.1016/j.chemosphere.2006.07.051

Gómez-Ramírez, P., Espín, S., Navas, I., Martínez-López, E., Jiménez, P., María-Mojica, P., ... & García-Fernández, A. J. (2020). Mercury and organochlorine pesticides in tissues of loggerhead sea turtles (*Caretta caretta*) stranded along the Southwestern Mediterranean coastline (Andalusia, Spain). *Bulletin of Environmental Contamination and Toxicology*, *104*(5), 559-567. https://doi.org/10.1007/s00128-020-02822-z

Gómez de Segura, A., Crespo, E. A., Pedraza, S. N., Hammond, P. S., & Raga, J. A., 2006. Abundance of small cetaceans in waters of the central Spanish Mediterranean. *Marine Biology*, *150*(1), 149-160. https://doi.org/10.1007/s00227-006-0334-0

Gross, T. S., Crain, D. A., Bjorndal, K. A., Bolten, A. B., & Carthy, R. R. (1995). Identification of sex in hatchling loggerhead turtles (*Caretta caretta*) by analysis of steroid concentrations in chorioallantoic/amniotic fluid. *General and comparative endocrinology*, *99*(2), 204-210. https://doi.org/10.1006/gcen.1995.1103

Grousset, F. E., Quetel, C. R., Thomas, B., Donard, O. F. X., Lambert, C. E., Guillard, F., & Monaco, A., 1995. Anthropogenic vs. lithogenic origins of trace elements (As, Cd, Pb, Rb, Sb, Sc, Sn, Zn) in water column particles: northwestern Mediterranean Sea. *Marine Chemistry*, *48*(3-4), 291-310. https://doi.org/10.1016/0304-4203(94)00056-J

Guerranti, C., Cau, A., Renzi, M., Badini, S., Grazioli, E., Perra, G., & Focardi, S. E., 2016. Phthalates and perfluorinated alkylated substances in Atlantic bluefin tuna (*Thunnus thynnus*) specimens from Mediterranean Sea (Sardinia, Italy): Levels and risks for human consumption. *Journal of Environmental Science and Health, Part B*, *51*(10), 661-667. https://doi.org/10.1080/03601234.2016.1191886

Guerzoni, S., Molinaroli, E., Rossini, P., Rampazzo, G., Quarantotto, G., De Falco, G., & Cristini, S., 1999. Role of desert aerosol in metal fluxes in the Mediterranean area. *Chemosphere*, *39*(2), 229-246. https://doi.org/10.1016/S0045-6535(99)00105-8

Halle, L. L., Palmqvist, A., Kampmann, K., & Khan, F. R., 2020. Ecotoxicology of micronized tire rubber: Past, present and future considerations. *Science of the Total Environment*, *706*, 135694. https://doi.org/10.1016/j.scitotenv.2019.135694

Hannas, B. R., Lambright, C. S., Furr, J., Howdeshell, K. L., Wilson, V. S., & Gray Jr, L. E., 2011. Dose-response assessment of fetal testosterone production and gene expression levels in rat testes following in utero exposure to diethylhexyl phthalate, diisobutyl phthalate, diisoheptyl phthalate, and diisononyl phthalate. *Toxicological Sciences*, *123*(1), 206-216. https://doi.org/10.1093/toxsci/kfr146

Hart, L. B., Beckingham, B., Wells, R. S., Alten Flagg, M., Wischusen, K., Moors, A., ... & Wirth, E., 2018. Urinary phthalate metabolites in common bottlenose dolphins (*Tursiops truncatus*) from Sarasota Bay, FL, USA. *GeoHealth*, *2*(10), 313-326. https://doi.org/10.1029/2018GH000146

Hidalgo-Ruz, V., Gutow, L., Thompson, R. C., & Thiel, M., 2012. Microplastics in the marine environment: a review of the methods used for identification and quantification. *Environmental science & technology*, *46*(6), 3060-3075. https://doi.org/10.1021/es2031505

Hlisníková, H., Petrovičová, I., Kolena, B., Šidlovská, M., & Sirotkin, A., 2020. Effects and mechanisms of phthalates' action on reproductive processes and reproductive health: a literature review. *International Journal of Environmental R esearch and Public Health*, *17*(18), 6811. https://doi.org/10.3390/ijerph17186811

Hotchkiss, A. K., Parks-Saldutti, L. G., Ostby, J. S., Lambright, C., Furr, J., Vandenbergh, J. G., & Gray Jr, L. E., 2004. A mixture of the "antiandrogens" linuron and butyl benzyl phthalate alters sexual differentiation of the male rat in a cumulative fashion. *Biology of reproduction*, 71(6), 1852-1861. https://doi.org/10.1095

Hu, X., Gu, Y., Huang, W., & Yin, D., 2016. Phthalate monoesters as markers of phthalate contamination in wild marine organisms. *Environmental pollution*, *218*, 410-418. https://doi.org/10.1016/j.envpol.2016.07.020

Hulley, P., 2015. *Lampanyctus crocodilus*. *The IUCN Red List of Threatened Species* 2015: e.T198618A15584685. https://dx.doi.org/10.2305/IUCN.UK.2015-4.RLTS.T198618A15584685.en. Accessed on 24 August 2022.

Jakimska, A., Konieczka, P., Skóra, K., & Namieśnik, J., 2011. Bioaccumulation of metals in tissues of marine animals, Part II: metal concentrations in animal tissues. *Polish Journal of Environmental Studies*, *20*(5).

Jambeck, J. R., Geyer, R., Wilcox, C., Siegler, T. R., Perryman, M., Andrady, A., ... & Law, K. L., 2015. Plastic waste inputs from land into the ocean. *Science*, *347*(6223), 768-771. https://doi.org/10.1126/science.1260352

Jamieson, A. J., Brooks, L. S. R., Reid, W. D., Piertney, S. B., Narayanaswamy, B. E., & Linley, T. D., 2019. Microplastics and synthetic particles ingested by deep-sea amphipods in six of the deepest marine ecosystems on Earth. *Royal Society open science*, *6*(2), 180667. https://doi.org/10.1098/rsos.180667

Kaska, Y., Celik, A., Bag, H., Aureggi, M., Özel, K., Elçi, A., ... & Elçi, L., 2004. Heavy metal monitoring in stranded sea turtles along the Mediterranean coast of Turkey. *Fresenius Environmental Bulletin*, *13*(8), 769-776.

Keller, J. M., 2013. 11 exposure to and effects of persistent organic pollutants. *The Biology of Sea Turtles, Volume III*, *3*, 285. ISBN: 978-1-4398-7308.

Kumar, N., Srivastava, S., & Roy, P., 2015. Impact of low molecular weight phthalates in inducing reproductive malfunctions in male mice: Special emphasis on Sertoli cell functions. *General and Comparative Endocrinology*, *215*, 36-50. https://doi.org/10.1016/j.ygcen.2014.09.012

Laglbauer, B. J., Franco-Santos, R. M., Andreu-Cazenave, M., Brunelli, L., Papadatou, M., Palatinus, A., ... & Deprez, T., 2014. Macrodebris and microplastics from beaches in Slovenia. *Marine pollution bulletin*, *89*(1-2), 356-366. https://doi.org/10.1016/j.marpolbul.2014.09.036

Lauriano, G., 2022. Stenella coeruleoalba (Mediterranean subpopulation) (errata version published in 2022). The IUCN Red List of Threatened Species 2022: e.T16674437A210833690. Accessed on 05 August 2022.

Lusher, A. L., Hernandez-Milian, G., O'Brien, J., Berrow, S., O'Connor, I., & Officer, R., 2015. Microplastic and macroplastic ingestion by a deep diving, oceanic cetacean: The True's beaked whale *Mesoplodon mirus*. *Environmental pollution*, *199*, 185-191. https://doi.org/10.1016/j.envpol.2015.01.023

Lusher, A. L., O'Donnell, C., Officer, R., & O'Connor, I., 2016. Microplastic interactions with North Atlantic mesopelagic fish. *ICES Journal of marine science*, *73*(4), 1214-1225. https://doi.org/10.1093/icesjms/fsv241

Lynch, J. M., 2018. Quantities of marine debris ingested by sea turtles: global meta-analysis highlights need for standardized data reporting methods and reveals relative risk. *Environmental science & technology*, *52*(21), 12026-12038. https://doi.org/10.1021/acs.est.8b02848

Maffucci, F., Caurant, F., Bustamante, P., & Bentivegna, F., 2005. Trace element (Cd, Cu, Hg, Se, Zn) accumulation and tissue distribution in loggerhead turtles (*Caretta caretta*) from the Western Mediterranean Sea (southern Italy). *Chemosphere*, *58*(5), 535-542. https://doi.org/10.1016/j.chemosphere.2004.09.032

Mansfield, K. L., Wyneken, J., Porter, W. P., & Luo, J., 2014. First satellite tracks of neonate sea turtles redefine the 'lost years' oceanic niche. *Proceedings of the Royal Society B: Biological Sciences*, *281*(1781), 20133039. https://doi.org/10.1098/rspb.2013.3039

Martinez-Ribes, L., Basterretxea, G., Palmer, M., & Tintoré, J., 2007. Origin and abundance of beach debris in the Balearic Islands. *Scientia Marina*, *71*(2), 305-314. ISSN: 0214-8358.

Masiá, A., Campo, J., Blasco, C., & Picó, Y., 2014. Ultra-high performance liquid chromatography–quadrupole time-of-flight mass spectrometry to identify contaminants in water: An insight on environmental forensics. *Journal of Chromatography A*, *1345*, 86-97. https://doi.org/10.1016/j.chroma.2014.04.017

Matiddi, M., Hochsheid, S., Camedda, A., Baini, M., Cocumelli, C., Serena, F., ... & de Lucia, G. A., 2017. Loggerhead sea turtles (*Caretta caretta*): A target species for monitoring litter ingested by marine organisms in the Mediterranean Sea. Environmental pollution, 230, 199-209. https://doi.org/10.1016/j.envpol.2017.06.054

Melendez-Pastor, I., Hernández, E. I., Navarro-Pedreño, J., Almendro-Candel, M. B., Gómez Lucas, I., & Jordán Vidal, M. M., 2021. Occurrence of Pesticides Associated with an Agricultural Drainage System in a Mediterranean Environment. *Applied Sciences*, *11*(21), 10212. https://doi.org/10.3390/app112110212

Molina, R., Manno, G., Lo Re, C., Anfuso, G., & Ciraolo, G., 2020. A Methodological Approach to Determine Sound Response Modalities to Coastal Erosion Processes in Mediterranean Andalusia (Spain). *Journal of Marine Science and Engineering*, *8*(3), 154. https://doi.org/10.3390/jmse8030154

Moreno-González, R., Campillo, J. A., & León, V. M., 2013. Influence of an intensive agricultural drainage basin on the seasonal distribution of organic pollutants in seawater from a Mediterranean coastal lagoon (Mar Menor, SE Spain). *Marine pollution bulletin*, *77*(1-2), 400-411. https://doi.org/10.1016/j.marpolbul.2013.09.040

Multisanti, C. R., Merola, C., Perugini, M., Aliko, V., & Faggio, C., 2022. Sentinel species selection for monitoring microplastic pollution: A review on one health approach. Ecological Indicators, 145, 109587. https://doi.org/10.1016/j.ecolind.2022.109587

Munari, C., Corbau, C., Simeoni, U., & Mistri, M., 2016. Marine litter on Mediterranean shores: analysis of composition, spatial distribution and sources in north-western Adriatic beaches. *Waste management*, *49*, 483-490. https://doi.org/10.1016/j.wasman.2015.12.010

Munaretto, J. S., May, M. M., Saibt, N., & Zanella, R., 2016. Liquid chromatography with high resolution mass spectrometry for identification of organic contaminants in fish fillet: screening and quantification assessment using two scan modes for data acquisition. *Journal of Chromatography A*, *1456*, 205-216. https://doi.org/10.1016/j.chroma.2016.06.018

Mrosovsky, N., Kamel, S., Rees, A. F., & Margaritoulis, D., 2002. Pivotal temperature for loggerhead turtles (*Caretta caretta*) from Kyparissia Bay, Greece. *Canadian Journal of Zoology*, *80*(12), 2118-2124. https://doi.org/10.1139/z02-204

Nelms, S. E., Duncan, E. M., Broderick, A. C., Galloway, T. S., Godfrey, M. H., Hamann, M., ... & Godley, B. J., 2016. Plastic and marine turtles: a review and call for research. *ICES Journal of Marine Science*, *73*(2), 165-181. https://doi.org/10.1093/icesjms/fsv165

Net, S., Delmont, A., Sempéré, R., Paluselli, A., & Ouddane, B., 2015. Reliable quantification of phthalates in environmental matrices (air, water, sludge, sediment and soil): A review. *Science of the Total Environment*, *515*, 162-180. https://doi.org/10.1016/j.scitotenv.2015.02.013

O'Hara, C. C., & Halpern, B. S., 2022. Anticipating the Future of the World's Ocean. *Annual Review of Environment and Resources*, *47*. https://doi.org/10.1146/annurev-environ-120120-053645

Paluselli (A), A., Fauvelle, V., Schmidt, N., Galgani, F., Net, S., & Sempere, R., 2018. Distribution of phthalates in marseille bay (NW Mediterranean Sea). *Science of the total environment*, *621*, 578-587. https://doi.org/10.1016/j.scitotenv.2017.11.306

Paluselli, A., Aminot, Y., Galgani, F., Net, S., & Sempere, R., 2018. Occurrence of phthalate acid esters (PAEs) in the northwestern Mediterranean Sea and the Rhone River. *Progress in Oceanography*, *163*, 221-231. https://doi.org/10.1016/j.pocean.2017.06.002

Pan, G., Hanaoka, T., Yoshimura, M., Zhang, S., Wang, P., Tsukino, H., ... & Takahashi, K., 2006. Decreased serum free testosterone in workers exposed to high levels of di-n-butyl phthalate (DBP) and di-2-ethylhexyl phthalate (DEHP): a cross-sectional study in China. *Environmental health perspectives*, *114*(11), 1643-1648. https://doi.org/10.1289/ehp.9016

Pasternak, G., Zviely, D., Ribic, C. A., Ariel, A., & Spanier, E., 2017. Sources, composition and spatial distribution of marine debris along the Mediterranean coast of Israel. *Marine Pollution Bulletin*, *114*(2), 1036-1045. https://doi.org/10.1016/j.marpolbul.2016.11.023

Pérez-Lucas, G., Vela, N., El Aatik, A., & Navarro, S., 2019. Environmental risk of groundwater pollution by pesticide leaching through the soil profile. *Pesticides-use and misuse and their impact in the environment*, 1-28. ISBN: 978-1-83880-047-5.

Plastics Europe, 2021. Plastics - the facts. Association of plastic manufacturers, 2021. http://ww.plasticseurope.org (Accessed August 2022)

Perrault, J., Wyneken, J., Thompson, L. J., Johnson, C., & Miller, D. L., 2011. Why are hatching and emergence success low? Mercury and selenium concentrations in nesting leatherback sea turtles (*Dermochelys coriacea*) and their young in Florida. *Marine pollution bulletin*, *62*(8), 1671-1682. https://doi.org/10.1016/j.marpolbul.2011.06.009

Prevenios, M., Zeri, C., Tsangaris, C., Liubartseva, S., Fakiris, E., & Papatheodorou, G., 2018. Beach litter dynamics on Mediterranean coasts: Distinguishing sources and pathways. *Marine pollution bulletin*, *129*(2), 448-457. https://doi.org/10.1016/j.marpolbul.2017.10.013Get

Rees, A. F., Alfaro-Shigueto, J., Barata, P. C. R., Bjorndal, K. A., Bolten, A. B., Bourjea, J., ... & Godley, B. J., 2016. Are we working towards global research priorities for management and conservation of sea turtles? *Endangered Species Research*, *31*, 337-382. https://doi.org/10.3354/esr00801

Revelles, M., Cardona, L., Aguilar, A., & Fernández, G., 2007. The diet of pelagic loggerhead sea turtles (*Caretta caretta*) off the Balearic archipelago (western Mediterranean): relevance of long-line baits. *Journal of the Marine Biological Association of the United Kingdom*, *87*(3), 805-813. https://doi.org/10.1017/S0025315407054707

Renau-Pruñonosa, A., García-Menéndez, O., Ibáñez, M., Vázquez-Suñé, E., Boix, C., Ballesteros, B. B., ... & Hernández, F., 2020. Identification of aquifer recharge sources as the origin of emerging contaminants in intensive agricultural areas. La Plana de Castellón, Spain. *Water*, *12*(3), 731. https://doi.org/10.3390/w12030731

Rian, M. B., Vike-Jonas, K., Gonzalez, S. V., Ciesielski, T. M., Venkatraman, V., Lindstrøm, U., ... & Asimakopoulos, A. G., 2020. Phthalate metabolites in harbor porpoises (*Phocoena phocoena*) from Norwegian coastal waters. *Environment international*, *137*, 105525. https://doi.org/10.1016/j.envint.2020.105525

Rios-Fuster, B., Alomar, C., González, G. P., Martínez, R. M. G., Rojas, D. L. S., Hernando, P. F., & Deudero, S., 2022. Assessing microplastic ingestion and occurrence of bisphenols and phthalates in bivalves, fish and holothurians from a mediterranean marine protected area. *Environmental Research*, 114034. https://doi.org/10.1016/j.envres.2022.114034

Rockström, J., Steffen, W., Noone, K., Persson, Å., Chapin III, F. S., Lambin, E., ... & Foley, J., 2009. Planetary boundaries: exploring the safe operating space for humanity. *Ecology and society*, *14*(2). http://www.jstor.org/stable/26268316

Romeo, T., Peda, C., Fossi, M. C., Andaloro, F., & Battaglia, P., 2016. First record of plastic debris in the stomach of Mediterranean lanternfishes. *Acta Adriatica: International Journal of Marine Sciences*, *57*(1), 115-122.

Ruckelshaus, M., Doney, S. C., Galindo, H. M., Barry, J. P., Chan, F., Duffy, J. E., ... & Talley, L. D., 2013. Securing ocean benefits for society in the face of climate change. *Marine Policy*, *40*, 154-159. https://doi.org/10.1016/j.marpol.2013.01.009

Sanjaume, E., & Pardo-Pascual, J. E., 2005. Erosion by human impact on the Valencian coastline (E of Spain). *Journal of Coastal Research*, 76-82. ISSN: 0749-0208.

Savoca, D., Arculeo, M., Barreca, S., Buscemi, S., Caracappa, S., Gentile, A., ... & Pace, A., 2018. Chasing phthalates in tissues of marine turtles from the Mediterranean Sea. *Marine pollution bulletin*, *127*, 165-169. https://doi.org/10.1016/j.marpolbul.2017.11.069

Schmidt, N., Castro-Jiménez, J., Oursel, B., & Sempéré, R., 2021. Phthalates and organophosphate esters in surface water, sediments and zooplankton of the NW Mediterranean Sea: Exploring links with microplastic abundance and accumulation in the marine food web. *Environmental Pollution*, *272*, 115970. https://doi.org/10.1016/j.envpol.2020.115970

Setälä, O., Fleming-Lehtinen, V., & Lehtiniemi, M., 2014. Ingestion and transfer of microplastics in the planktonic food web. *Environmental pollution*, *185*, 77-83. https://doi.org/10.1016/j.envpol.2013.10.013

Sharma, A., Mollier, J., Brocklesby, R. W., Caves, C., Jayasena, C. N., & Minhas, S., 2020. Endocrine-disrupting chemicals and male reproductive health. *Reproductive medicine and biology*, *19*(3), 243-253. https://doi.org/10.1002/rmb2.12326

Sharma, S., Sharma, V., & Chatterjee, S., 2021. Microplastics in the Mediterranean Sea: sources, pollution intensity, sea health, and regulatory policies. *Frontiers in Marine Science*, *8*, 634934. https://doi.org/10.3389/fmars.2021.634934

Sohn, J., Kim, S., Koschorreck, J., Kho, Y., & Choi, K., 2016. Alteration of sex hormone levels and steroidogenic pathway by several low molecular weight phthalates and their metabolites in male zebrafish (*Danio rerio*) and/or human adrenal cell (H295R) line. *Journal of hazardous materials*, *320*, 45-54. https://doi.org/10.1016/j.jhazmat.2016.08.008

Standora, E. A., & Spotila, J. R., 1985. Temperature dependent sex determination in sea turtles. *Copeia*, 711-722. https://doi.org/10.2307/1444765

Storelli, M. M., Ceci, E., & Marcotrigiano, G. O., 1998. Distribution of heavy metal residues in some tissues of *Caretta caretta* (Linnaeus) specimens beached along the Adriatic Sea (Italy). *Bulletin of Environmental Contamination and Toxicology*, *60*(4), 546-552.

Storelli, M. M., Storelli, A., D'addabbo, R., Marano, C., Bruno, R., & Marcotrigiano, G. O., 2005. Trace elements in loggerhead turtles (*Caretta caretta*) from the eastern Mediterranean Sea: overview and evaluation. *Environmental Pollution*, *135*(1), 163-170. https://doi.org/10.1016/j.envpol.2004.09.005

Suaria, G., & Aliani, S., 2014. Floating debris in the Mediterranean Sea. *Marine pollution bulletin*, *86*(1-2), 494-504. https://doi.org/10.1016/j.marpolbul.2014.06.025

Schwacke, L. H., Gulland, F. M., & White, S., 2013. Sentinel species in oceans and human health. In *Environmental toxicology* (pp. 503-528). Springer, New York, NY. https://doi.org/10.1007/978-1-4419-0851-3

Thompson, R. C., Olsen, Y., Mitchell, R. P., Davis, A., Rowland, S. J., John, A. W., ... & Russell, A. E., 2004. Lost at sea: where is all the plastic? *Science*, *304*(5672), 838-838. https://doi.org/10.1126/science.1094559

Tomas, J., Aznar, F. J., & Raga, J. A., 2001. Feeding ecology of the loggerhead turtle *Caretta caretta* in the western Mediterranean. *Journal of Zoology*, *255*(4), 525-532. https://doi.org/10.1017/S0952836901001613

Tomás, J., Guitart, R., Mateo, R., & Raga, J. A., 2002. Marine debris ingestion in loggerhead sea turtles, *Caretta caretta*, from the Western Mediterranean. Marine pollution bulletin, 44(3), 211-216. https://doi.org/10.1016/S0025-326X(01)00236-3

United Nations Environmental Programme/Mediterranean Action Plan and Plan Bleu., 2020. State of the environment and development of the Mediterranean. Nairobi. ISBN 978-92-807-3796-7.

Villarrubia-Gómez, P., Cornell, S. E., & Fabres, J., 2018. Marine plastic pollution as a planetary boundary threat–The drifting piece in the sustainability puzzle. *Marine policy*, *96*, 213-220. https://doi.org/10.1016/j.marpol.2017.11.035

Vlachogianni, T., Fortibuoni, T., Ronchi, F., Zeri, C., Mazziotti, C., Tutman, P., ... & Scoullos, M., 2018. Marine litter on the beaches of the Adriatic and Ionian Seas: An assessment of their abundance, composition and sources. *Marine pollution bulletin*, *131*, 745-756. https://doi.org/10.1016/j.marpolbul.2018.05.006

Wang, J., Tan, Z., Peng, J., Qiu, Q., & Li, M., 2016. The behaviors of microplastics in the marine environment. *Marine Environmental Research*, *113*, 7-17. https://doi.org/10.1016/j.marenvres.2015.10.014

Wang, W., Gao, H., Jin, S., Li, R., & Na, G., 2019. The ecotoxicological effects of microplastics on aquatic food web, from primary producer to human: A review. *Ecotoxicology and environmental safety*, *173*, 110-117. https://doi.org/10.1016/j.ecoenv.2019.01.113

Wyneken, J., & Lolavar, A., 2015. Loggerhead sea turtle environmental sex determination: implications of moisture and temperature for climate change based predictions for species survival.

Journal of Experimental Zoology Part B: Molecular and Developmental Evolution, *324*(3), 295-314. https://doi.org/10.1002/jez.b.22620

Yipel, M., Tekeli, İ. O., İşler, C. T., & Altuğ, M. E., 2017. Heavy metal distribution in blood, liver and kidneys of Loggerhead (*Caretta caretta*) and Green (*Chelonia mydas*) sea turtles from the Northeast Mediterranean Sea. *Marine pollution bulletin*, *125*(1-2), 487-491. https://doi.org/10.1016/j.marpolbul.2017.08.011

Yusà, V., Fernández, S. F., Dualde, P., López, A., Lacomba, I., & Coscollà, C., 2022. Exposure to non-persistent pesticides in the Spanish population using biomonitoring: A review. *Environmental Research*, *205*, 112437. https://doi.org/10.1016/j.envres.2021.112437

PROGRAMA DE DOCTORAT
EN BIODIVERSITAT I
BIOLOGIA EVOLUTIVA 3101

Facultat de
Ciències Biològiques

VNIVERSITAT DE VALÈNCIA