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The role of microbial ecology during biogas production from renewable energy sources. Characterizing microbial community structures in bioenergy production systems for future water resource recovery facilities.

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CERTIFICAN:

que la presente memoria, titulada "**The role of microbial ecology during biogas production from renewable energy sources. Characterizing microbial community structures in bioenergy production systems for future water resource recovery facilities**", corresponde al trabajo realizado bajo su dirección por **DÑA. NÚRIA ZAMORANO LÓPEZ**, para su presentación como tesis doctoral en el programa de doctorado en Ingeniería Química, Ambiental y de Procesos de la Universitat de València.

Y para que conste firma/n el presente certificado en Valencia, a 2 de octubre de 2019

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Abstract

Microbiology has a fundamental role in the integration of nutrients, energy and water recovery in biological wastewater treatment processes. The knowledge about key microbial groups composition, structure, dynamics, and ecology, will improve the comprehension of processes integrated into future Water Resource Recovery Facilities (WRRF) and could be helpful to optimize the anaerobic systems for bioenergy recovery from renewable sources. The combination of anaerobic membrane bioreactors (AnMBR) for sewage treatment and membrane photobioreactors (MPBR) for microalgae cultivation produces high-quality reclaimed water and is a sustainable solution on a circular economy frame. Also, the enhance of hydrolytic groups in microbial communities is a feasible strategy to boost biomethanization when using renewable sources that are produced in municipalities (e.g. food waste) or during the treatment of wastewater (microalgae, sewage sludge).

This work evaluates the microbial ecology of seven anaerobic reactors for bioenergy recovery from renewable sources during performance optimization. Massive sequencing of 16S rDNA biomarker has been applied in these systems to detect the influence of the operational parameters on the bioreactor microbiology. The studies have been carried out at both the laboratory and the pilot plant scales. The complexity of the information retrieved through high-throughput sequencing has required the development of bioinformatics and biostatistics knowledge. The application of multivariate analysis techniques has allowed the full comprehension of the effect of operational parameter selection such as temperature, inoculum source, hydraulic and solids retention time, organic loading rate and influent composition. Besides, different bioreactor configurations have been explored, including the AnMBR because of its potential integration in future WRRF.

This study demonstrates that the temperature is the most influencing parameter over microbial communities. The most remarkable mesophilic phyla of anaerobic systems were 15-30% *Chloroflexi*, 14-27%

Proteobacteria, 2-19% *Bacteroidetes*, 2-15% *Firmicutes*, and 1-7% *Synergistes*; and 6-44% *Thermotoga* and 17-32% *Firmicutes* for thermophilic systems. Mesophilic systems for microalgae degradation through digestion or co-digestion share 57% of their microbial diversity. The differences were mainly attributed to solids (SRT) and hydraulic (HRT) retention times. The rDNA and rRNA sequencing strategy is especially recommended for thermophilic systems to remove the background groups associated with the feedstock biomass.

Finally, it is concluded in this work that the use of acclimated communities at high SRT using AnMBR systems is a better alternative than the use of exogenous hydrolytic consortia since they are more resistant to changes in the operational conditions. Moreover, both *Scenedesmus* and *Chlorella* microalgae can be degraded by similar communities without pre-treatments.

Resum

La Microbiologia té un rol fonamental en la integració de processos per a la recuperació de nutrients, energia i aigua durant el tractament biològic de l'aigua residual. La identificació dels grups microbiològics claus, així com de les seues dinàmiques, ecologia i estructures microbianes millorarà l'enteniment dels processos que integren les futures plantes de recuperació de recursos, conegudes per les seues sigles en anglès com *Water Resource Recovery Facilities* (WRRF). Aquest coneixement podria ser de gran utilitat durant l'optimització de sistemes anerobis que recuperen energia de fonts de biomassa renovables. Fins a l'actualitat, diferents estudis han demostrat que la combinació de bioreactors anaerobis de membranes (AnMBR) per al tractament de l'aigua residual i els fotobioreactors de membranes per al cultiu de microalgues, produeixen un efluent d'alta qualitat i són una opció sostenible, que està emmarcada en el concepte d'economia circular. A més a més, l'augment del potencial hidrolític de les comunitats microbianes ha demostrat ser una prometedora estratègia per a incrementar el potencial de recuperació de metà a partir de fonts re biomassa renovables generades en àrees municipals (com la fracció orgànica dels residus sòlids urbans) o durant el tractament de l'aigua residual (biomassa de microalgues o fangs de depuradores).

Aquest treball avalua l'ecologia microbiana d'un total de set reactors anaerobis que recuperen bioenergia a partir de fonts de biomassa renovables. La seqüenciació massiva del biomarcador de microorganismes procariotes (gen 16S rDNA) ha estat aplicat en tots els sistemes per a detectar la influència dels paràmetres operacionals sota l'ecologia microbiana dels bioreactors durant l'optimització del procés. Els estudis han sigut elaborats a escala de laboratori i de planta pilot. La complexitat d'aquests estudis ha motivat el desenvolupament d'una metodologia en aquest treball per a l'anàlisi de dades de bioinformàtica i el seu posterior tractament amb tècniques de bioestadística. En aquest context, l'aplicació de tècniques d'anàlisi multivariant ha permès comprendre l'efecte dels paràmetres operacionals claus com són la temperatura, l'inòcul, els temps de retenció hidràulic i cel·lular, la velocitat de càrrega orgànica i la composició de

l'afluent. A més a més, s'han comparat diverses configuracions dels bioreactors, incloent-hi el reactor AnMBR pel seu alt potencial d'integració en les futures WRRF.

En aquest estudi es demostra que el rang de temperatura és el paràmetre amb la influència més gran sota les comunitats microbianes. Els *phyla* més destacats dels sistemes mesofílics i les seues abundàncies relatives han sigut 15-30% *Chloroflexi*, 14-27% *Proteobacteria*, 2-19% *Bacteroidetes*, 2-15% *Firmicutes*, i 1-7% *Synergistes*. En els sistemes termofílics cal destacar la presència de 17-32% *Firmicutes* i 6-44% *Thermotoga*. A més a més, cal destacar que els sistemes mesofílics de degradació de microalgues compartiren un 57% de la seua diversitat microbiana independentment de l'adició o no d'un cosubstrat i que les diferències trobades van ser atribuïdes als temps de retenció hidràulic i cel·lular. L'anàlisi de rDNA i rRNA es recomana per a sistemes termofílics amb el fi d'eliminar els grups de microorganismes de fons associats a la diversitat microbiana intrínseca de la biomassa.

Finalment, en aquest treball es conclou que l'ús de comunitats adaptades és una millor alternativa a l'ús de cultius externs hidrolítics, en tindre una major resistència davant canvis en les condicions operacionals durant l'optimització dels processos de producció de biogàs. A més a més, les microalgues *Scenedesmus* i *Chlorella* que creixen en efluents anaerobis poden ser degradades per comunitats microbianes similars i sense l'aplicació de pretractaments.

Resumen

La Microbiología tiene un rol fundamental en la integración de procesos para la recuperación de nutrientes, energía y agua durante el tratamiento biológico del agua residual. La identificación de los grupos microbiológicos clave, así como de sus dinámicas, ecología y estructuras microbianas, mejorará el entendimiento de los procesos que integran las futuras plantas de recuperación de recursos, conocidas por sus siglas en inglés como *Water Resource Recovery Facilities* (WRRF). Este conocimiento podría ser de gran utilidad durante la optimización de sistemas anaerobios que recuperan energía a partir de fuentes de biomasa renovables. Hasta la fecha, diferentes estudios han demostrado que la combinación de los biorreactores anaerobios de membranas (AnMBR) para el tratamiento de agua residual y los fotobiorreactores de membranas para el cultivo de microalgas producen un efluente de alta calidad y son una opción sostenible, enmarcada en un concepto de economía circular. Además, el aumento del potencial hidrolítico de las comunidades microbianas ha demostrado ser una prometedora estrategia para incrementar el potencial de recuperación de metano a partir de fuentes de biomasa renovables, que se generan en las áreas municipales (como la fracción orgánica de los residuos sólidos urbanos) o durante los tratamientos del agua residual (biomasa de microalgas o fangos de depuradora).

Este trabajo evalúa la ecología microbiana de un total de siete reactores anaerobios empleados en la recuperación de bioenergía a partir de fuentes de biomasa renovables. La secuenciación masiva del biomarcador de microorganismos procariotas (gen 16S rDNA) ha sido aplicada en todos estos sistemas para detectar la influencia de los parámetros operacionales sobre la ecología microbiana de los biorreactores durante la optimización del proceso. Los estudios han sido llevados a cabo a escala de laboratorio y de planta piloto. La complejidad de estos estudios de secuenciación ha motivado el desarrollo de una metodología en este trabajo para el análisis de datos de bioinformática y su posterior tratamiento con técnicas de bioestadística. En este contexto, la aplicación de técnicas de análisis multivariante ha permitido comprender el efecto de parámetros

operacionales clave tales como la temperatura, la fuente de inóculo, los tiempos de retención hidráulico y celular, la velocidad de carga orgánica y la composición del afluente. Además, se han comparado diversas configuraciones de reactores, incluyendo el reactor AnMBR por su alto potencial de integración en las futuras WRRF.

En este estudio se demuestra que la temperatura es el parámetro con la mayor influencia sobre las comunidades microbianas. Los *phyla* más abundantes en condiciones mesofílicas fueron 15-30% *Chloroflexi*, 14-27% *Proteobacteria*, 2-19% *Bacteroidetes*, 2-15% *Firmicutes*, y 1-7% *Synergistes*. En los sistemas termofílicos destacaron 17-32% *Firmicutes* y 6-44% *Thermotoga*. Cabe destacar que los sistemas mesofílicos de degradación de microalgas compartían un 57% de su diversidad microbiana y que las diferencias observadas se atribuían a los tiempos de retención hidráulico y celular. El análisis de rDNA y rRNA se recomienda para sistemas termofílicos con el fin de eliminar los grupos de microorganismos de fondo que se asocian a la diversidad microbiana intrínseca de la biomasa.

Finalmente, en este trabajo se concluye que el uso de comunidades aclimatadas a altos tiempos de retención celular en reactores AnMBR es una mejor alternativa que el uso de cultivos externos hidrolíticos, ya que tienen una mayor resistencia ante cambios en las condiciones operacionales. Además, las microalgas *Scenedesmus* y *Chlorella,* que crecen en efluentes anaerobios, pueden ser degradadas por comunidades microbianas sin aplicar pretratamientos.

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1. Introduction

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1.1.Background

Water and life could be considered synonyms. As the current population of the planet understands, there would be no life without this essential element. Water management is thus critical to maintain a balance between the availability of this resource and its usage. On this basis, good environmental practices need to be addressed worldwide to deal with the current water scarcity and the environmental and sociological needs.

After the industrial revolution, the anthropogenic pressure over the environment has increasingly produced several negative impacts disturbing the lithosphere, biosphere, hydrosphere and atmosphere. The unsustainable use of water to satisfy the global population demands has induced dramatical ecological effects in the environment. Furthermore, water flows of the inner land and coastal areas close to municipalities or industrial locations have been also affected by the anthropogenic pressure. Water pollution is the result of the accumulation of certain substances that are toxic over a determined level. They are mainly originated by human practices, as natural sources rarely achieve high concentrations and are widely spread in the environment. The contaminants that are commonly released through anthropogenic activities can be classified according to their chemical nature in organic and inorganic pollutants. Their composition varies depending on their source, being those generated in urban areas commonly biodegradables while others resulting from industrial activities are more complex and often contain hazardous elements with low biodegradability. In response to the water pollution problem, several countries and associations have introduced different environmental protection measures in their policies at the end of the 20th Century. In June 1992 took place the United Nations Conference on Environment and Development (UNCED) and proclaimed that:

"Human beings are at the center of concerns for sustainable development. They are entitled to a healthy and productive life in harmony

with nature". Principle 1, The United Nations Conference on Environment and Development, (1992).

In the European Union (EU) the Water Framework Directive (2000/60/EC), adopted in 2000 by the belonging countries provides protection to water sources. This policy was incorporated to the Spanish legal system through the regulation RD 907/2007, entitled "*Reglamento de planificación hidrológica*". After that and during the 2000s other related directives have expanded the protection to water flows through the regulation of wastewater treatment also attending to emergent pollutants, coliforms and pathogens in wastewater (91/271/EEC), nitrates released from agricultural sources (91/676/EEC) and the use of sewage sludge in agriculture (86/278/EEC). More recently, as a response to the European Citizen's Initiative "Right2Water" the European Commission (EC) has presented a new proposal to update the Water Framework Directive after almost two decades. This initiative searches the guarantee of water and sanitation for all Europe, the no liberalization of water services and the global access to both water and sanitation.

Engineering processes applied to wastewater treatment have contributed to preserve the ecological and chemical status of freshwater flows and coasts, increasing the healthiness of the worldwide population, ecosystems and biodiversity during the last decades, assessing the protection levels pursued by the abovementioned policies. Nevertheless, the energetic cost of the conventional processes for wastewater treatment has called into question the sustainability of these processes despite their environmental and socioeconomical benefits. Accordingly, the scientific community has made a huge effort to seek for new energy supplies, as nowadays the main energy source still depends on fossil-fuels availability. This leads into a global problematic scenario, since the scarcity of carbon-based fuels is clear and recent studies are predicting their lack to cover the current society needs by 2050 (McGlade and Ekins, 2015). The pursuit of new sustainable and renewable energy sources pointed out the generation of energy from biomass and more specifically wastes (e.g. municipal solid organic wastes, sewage, agricultural disposures, industrial wastes, among others) to achieve a double

benefit. Generally, the management of energy production and water treatment has been performed separately. However, on the basis of a circular economy a water-energy nexus also involving waste management could provide new solutions, decreasing our dependence to conventional fossil fuels, boosting energy production from wastes and mitigating the energetic and economic demand of conventional waste treatments.

A long-term objective has been set by the EU-countries to develop a competitive economy in terms of energy production using low-carbon dioxide emission resources by 2050 and to promote a climate neutral economy. More precisely, three main targets have been proposed for 2020: (i) to reduce at least the 20% of greenhouse gas emissions (from 1990 levels), (ii) 20% presence of renewable energy sources and (iii) 20% improvement in energy efficiency. These targets are expected to be increased by 2030 in an additional 20%, 12% and 12.5%, correspondingly. The fulfilment of these targets requires an economic and social transformation and would be only possible with a paradigm change of our current technologies.

The circular economy concept proposed in 2014 by the Water Environment Federation (WEF) focuses on the products and benefits of water treatment rather than waste coming into facilities. The recovery of energy as well as nutrients, biosolids and water is the main goal of future Water Resource Recovery Facilities (WRRF), that replace the conventional Wastewater Treatment Plants (WTTP) locations. WRRF layouts could include an anaerobic system that would allow bioenergy generation from different wastes. This results from a biological process of organic matter degradation in absence of oxygen, known as anaerobic digestion (AD). The final product of this process is called biogas and can be turned into energy, since it contains 40-70% of methane, being the other gas components carbon dioxide and traces (hydrogen and hydrogen sulfide). As Table 1.1 shows, biogas has a calorific value of 6-6.5 $kWh \cdot m^{-3}$ (5000 $kcal \cdot m^{-3}$) and if ignited results in a clean burn similar to liquefied petroleum gas or compressed natural gas. Also, the AD of organic matter results in a high mineralization level of the initial biomass.

Table 1.1. Fuel calorific values and their equivalence to biogas mass. Source: Eidg. Anstalt für Wasserversorgung, Abwasserreinigung & Gewässerschutz (EAWAG).

Fuel source	Approximate calorific value ($kWh \cdot m^{-3}$)	Equivalent biogas (kg)
Biogas	6.0-6.5	
Diesel, kerosene	12.0	0.50
Wood	4.5	1.30
Cow dung	5.0	1.20
Plant residues	4.5	1.30
Hard coal	8.5	0.70
Propane	25.0	0.24
Natural gas	10.6	0.60
Liquified petroleum gas	26.1	0.20

Developing and industrialized countries have been attracted by the benefits of AD, *i.e.* biogas and a nutrient-rich digestate generation. It was not until 1982 that AD was applied to the treatment of wastewater, with the invention of septic tanks(Abbasi *et al.*, 2012; McCarty, 1982). Since then, anaerobic digestion has been used to stabilize high-strength biodegradable wastes. In the EU, biogas plants are widely extended and have especially reached during the last decade up to 17,783 (European Biogas Association, 2017). Germany is on the top of the list with the use of agriculture residues as the main feedstock for anaerobic digestion plants (Figure 1.1).

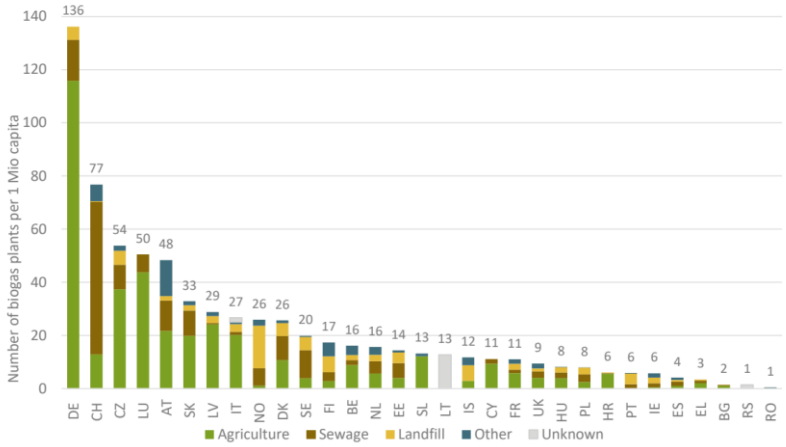

Figure 1.1. Number of biogas plants per 1 Mio capita in European countries according to the European Biogas Association (EBA, 2017).

Although AD was an attractive technology, some requirements challenged their application in WWTPs: (i) maintenance of an operational temperature range of 30-40ºC (mesophilic) or 50-60ºC (thermophilic), (ii) achievement of high solids retention time (SRT) to mitigate the low settleability and slow kinetics of the anaerobic microorganisms and (iii) need of a post-treatment stage to remove the high nutrient concentration that is released after organic matter mineralization. For these reasons, conventional activated sludge systems (CAS), an oxygen-dependent process, have been preferred over AD systems in WWTPs. Moreover, application of AD technology has been traditionally limited to the stabilization of the sludge streams generated in the CAS-WWTP, their co-digestion with other wastes from municipalities or industrial close areas and high-strength industrial wastewaters. However, 0.6 kWh are consumed per m^3 of wastewater treated through CAS according to Owen *et al.* (1982). It should be highlighted that half of this value only refers to the electrical energy demanded by aeration. Thus, the high energy consumption associated to the aeration requirements of CAS has promoted the search of alternatives for domestic water treatment in the last decade. Besides the energetic aspects, CAS-WWTPs are limiting the chance to recover valuable resources from wastewater, such as nutrients and high nutrient content reclaimed water that could be used for fertirrigation purposes. For example, nitrogen is commonly loss to the atmosphere in a post-stage to CAS that applies nitrification/denitrification to enhance nitrogen removal. On the contrary, AD could achieve a net energy production while meeting stringent effluent standards with the additional benefit of generating a nutrient rich solid and liquid stream. Hence, implementation of AD systems is an appealing alternative towards more sustainable systems for not only remove pollutants from sewage, but also maximize resource recovery (McCarty *et al.*, 2011). This would help the scientific community and the worldwide population to move forward a circular economy scenario.

1.2.The anaerobic digestion of organic matter

AD naturally occurs in anaerobic environments such as marshes, sediments and ruminant animals (Thauer *et al.*, 2008) but also in anthropogenic locations with decaying organic matter in absence of oxygen.

In 1868, the biologist and chemist Bechamp related this process to a combination of microbial interactions, represented in Figure 1.2.

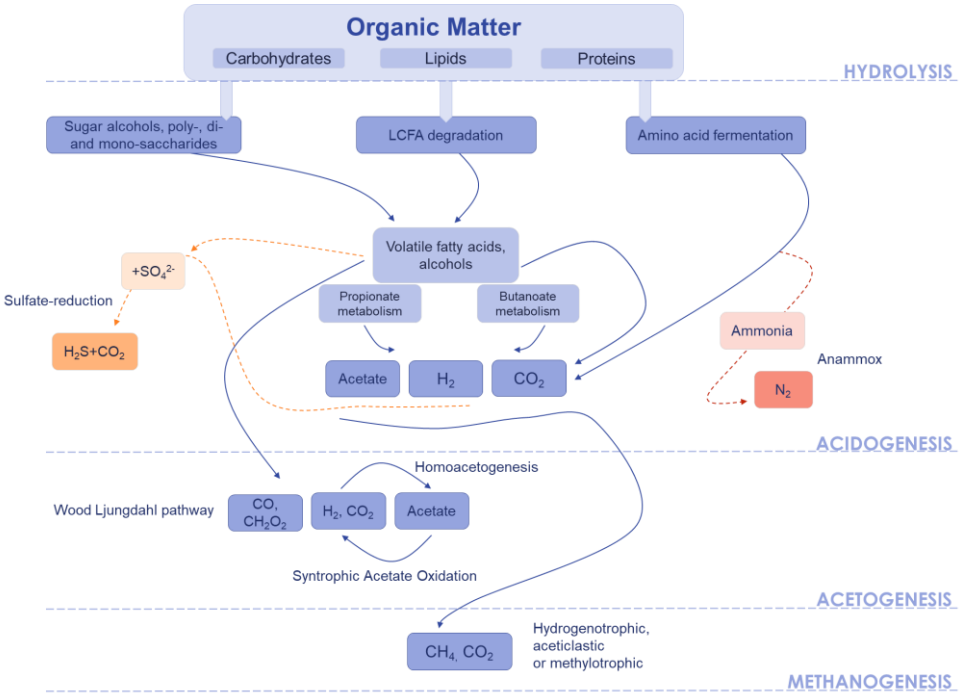

Figure 1.2. Diagram of organic matter anaerobic digestion. Source: adapted from (Angelidaki *et al.*, 2018).

Four different stages represent AD from a lineal perspective, starting with the more complex organic matter until the most reduced carbon compounds: methane and carbon dioxide. The first stage consists on the hydrolysis of carbohydrates and lipids, as well as the disruption of protein structures. Extracellular enzymes released by different microorganisms including fungi and bacteria catalyze this hydrolysis. After polysaccharides cleave, dimers and monomers of sugars are released and available for fermentative (or acidogenic) bacteria. Simple and soluble products released from carbohydrates and proteins are then fermented by a wide diversity of bacteria. As a result, reduced forms of carbon (*i.e.* carbon dioxide) and hydrogen are produced. Besides, a heterogeneous mixture of volatile fatty acids (VFA) and solvents is also released being butyrate, propionate and acetate the most commons. However, different fermentation pathways lead

to different by-products. This is specific to the diversity of microorganisms involved and the thermodynamics (Cabrol *et al.,* 2017). The VFA are further reduced in the acetogenesis stage into acetate and carbon dioxide, commonly through the Wood Ljungdahl pathway which is also known as the reductive acetyl-coenzyme A (Acetyl-CoA) pathway. Carbon monoxide and formic acid are produced in this process. Moreover, lipid degradation results in glycerol and long-chain fatty acids (LCFA) that are further degraded through the β-oxidation pathway. This process requires the syntrophic action of different microorganisms and is limited by the hydrogen partial pressure. Acetogenic bacteria preferentially produce acetic acid at high hydrogen concentrations, pH over 7 and high temperatures. On the contrary, this pathway channels to hydrogen and carbon dioxide, especially with increasing hydrogen concentration, pH and low temperatures (Thauer *et al.*, 2008). Hence, the release of hydrogen is key during AD since it regulates many other different intermediate reactions such as sulfate-reduction, syntrophic acetate oxidation (SAO), homoacetogenesis and methanogenesis (Figure 1.2).

Methanogenesis is the final stage of AD and is mainly linked to archaea microorganisms, which are capable to reduce the carbon dioxide into methane using hydrogen as an electron donor (hydrogenotrophic methanogens), cleave the acetate (aceticlastic methanogens) or catabolize methylated compounds including methanol, methylamines and methylsulfides (methylotrophic methanogens) into methane (Table 1.2). Finally, other anaerobic processes occur during AD, such as denitrification and sulfate reduction. The first consists on the conversion of ammonia into nitrogen, a process named anammox which is carried out by different bacteria (Jetten *et al.*, 1999). Sulfate reduction has been observed in both Bacteria and Archaea domains and consists on the metabolization of a huge range of compounds including hydrogen and different VFAs, using sulfate forms as electron acceptors.

Table 1.2. Gibbs-free energy changes for the different methane generation pathways.

Methane generation pathway	ΔG° ** 25°C	ΔG° ** 55°C	Reference
CO_2 reduction			
$HCO_3^- + 4H_2 + H^+ \rightarrow CH_4 + 3H_2O$	-175.32	-167.56	(Yang *et al.*, 2016)
Aceticlastic			
$4\ CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$	-103.7	n.d.	(Thauer *et al.*, 2008)
Formate			
$4HCOO^- + H_2O + H^+ \rightarrow CH_4 + 3HCO_3^-$	-170.84	-172.51	(Yang *et al.*, 2016)
*Methylotrophic**			
$H_2 + CH_3OH \rightarrow CH_4 + H_2O$	-112.5	n.d.	(Lang *et al.*, 2015)

*Methanol is here used as a reference substrate. **Expressed in $kJ \cdot molCH_4^{-1}$ unit.

1.3. Engineering solutions towards a circular economy waste management

1.3.1. Bioenergy recovery using anaerobic digesters

Engineering systems mimic the AD process to allow a fast and controlled conversion of organic matter into bioenergy *i.e.* biogas. Anaerobic digesters have been designed to replicate the ecological niche of anaerobic microorganisms involved in the four stages of AD (single stage AD) or separated the first stages from methanogenesis (two-stage AD). There are many configurations of conventional anaerobic treatments such as Continuous Stirred Tank Reactor (CSTR), Anaerobic Baffled Reactor (ABR), up-flow anaerobic sludge blanket reactor (UASB), the expanded granular sludge bed (EGSB) and the Anaerobic Membrane Reactor (AnMBR). An indispensable characteristic of these systems is that they must guarantee the retention of the microorganisms over time. Since anaerobic microorganisms have low kinetics, this was the main challenge during the design of bioengineering systems for wastewater treatment.

a. <u>High solids retention anaerobic bioreactors</u>

Implementation of AD for biological treatment of wastewater was prompted by Lettinga *et al.* (1980) with the design of the UASB. This system allowed the separation of the solid and the liquid phases in the reactor using a specific biomass *i.e* granular sludge. In this way, sedimentation of a granular sludge blanket was possible and consequently enhanced the SRT of the system. This overcame one of the main barriers of AD, as high SRTs are required to allow anaerobic microorganisms to thrive in the system despite

their slow growth kinetics. However, biological treatment in an UASB system still requires a secondary stage for nutrient removal due to the high mineralization capacity of anaerobic processes. Furthermore, methane production in UASB systems is sensitive to cold and low-strength wastewaters (Rebac *et al.,* 1999). Also, it has been well reported that gradients can be established in these systems limiting their application wastewaters that can release high concentrations of free ammonia or LCFA, potential inhibitors of methanogenesis. Additional problems when operating granular systems are: (i) disintegration of the granular structure, appearance of (ii) fluffy or (iii) hollow granules and (iv) precipitation of inorganic compounds (van Lier *et al.,* 2001). Besides, high concentration of suspended solids in the influent might also inhibit granulation resulting in a low performance of UASB systems. The difficulties associated to the use of a granular sludge can be overcome achieving a complete solid-liquid separation in anaerobic systems.

b. <u>The Anaerobic Membrane Bioreactor (AnMBR)</u>

The AnMBR results from the combination of membrane and anaerobic (Figure 1.3). This system allows a total retention of the biomass regardless its granulation properties, producing high quality effluents with a high level of disinfection (Dereli *et al.,* 2012). One of the major benefits of AnMBRs is the decoupling of SRT from hydraulic retention time (HRT), which prevents the washout of anaerobic microorganisms and allows the reduction of the reactor volume, as higher influent rates can be treated in shorter times. It was not until the 2000s that the AnMBR was implemented for biological treatment although it was designed in the late 70s (Grethlein, 1978). The development of more efficient membranes with long life cycles and lower costs was critical in this context, since the main drawback of AnMBR is the investment cost. Different configurations of the AnMBR can be found in the literature as Figure 1.3a, 1.3b, and 1.3c shows.

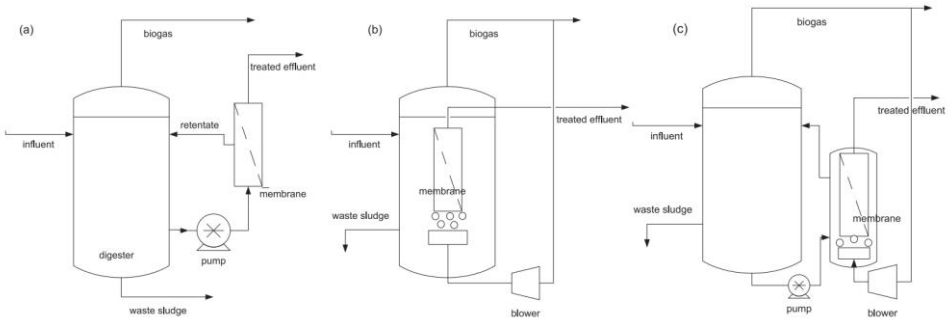

Figure 1.3. AnMBR configurations: (a) side stream membrane bioreactor (AnsMBR), (b) immersed membrane bioreactor (AnMBR) and (c) combination of a and b configurations. Source: Robles *et al.* (2018).

Nowadays, membrane technology research area is expanding with the implementation of innovative filtration separation systems and support materials (Jankhah, 2018; Le and Nunes, 2016). Thus, it should be expected that the role of the AnMBR in bioengineering processes for resource recovery from wastewater would be even more important in the next years. Nevertheless, innovations in the AnMBR would be needed to overcome some of the main limitations of this technology despite of its numerous benefits, which are listed in Table 1.3. The main barrier of AnMBRs is the loss of dissolved methane in the effluent when operating at low temperatures, but recent advances focused on methane recovery are moving AnMBR to a more positive-energy scenario (Cookney *et al.*, 2016).

During the last decade, different waste streams have been efficiently treated using AnMBRs such as municipal wastewaters (Ozgun *et al.*, 2013; Skouteris *et al.*, 2012), food waste (Galib *et al.*, 2016) or even the combination of both influents (Moñino *et al.*, 2016). More recently, complex feedstocks that commonly inhibit anaerobic processes have been also degraded in AnMBRs. For example, Kamali *et al.* (2016) optimized methane production from pulp and paper mill wastes using this technology. More recently, Muñoz Sierra *et al.* (2018) reported the use of this system to treat a phenolic wastewater, while Cheng *et al.* (2018) demonstrated that antibiotics can be also treated in AnMBRs although this practice was reported to aggravate membrane fouling. Precisely, fouling is another of the challenges of AnMBRs, as it hampers the operation of these systems

(Skouteris *et al.*, 2012). In fact, since membrane fouling and cleaning is a classical key issue regarding AnMBRs (Aslam *et al.*, 2018, 2017), its mitigation is needed to reduce the membrane cleaning associated costs (Wang *et al.*, 2014). However, membrane biofouling can have a positive effect over AD. The development of a membrane biofilm differs from bulking processes and can be beneficial to retain niche microorganisms that otherwise would be washed out from the system (Robles *et al.*, 2018). This is a key aspect to expand the application of AnMBRs to better capture the full energy, water and nutrient resource potential from wastewater (McCarty *et al.*, 2011).

Table 1.3. Comparison between conventional aerobic and anaerobic treatment versus AnMBR technology. Source: adapted from Lin *et al.* (2013).

Feature	**Aerobic, conventional**	**Anaerobic, conventional**	**AnMBR**
Organic removal efficiency	High	High	High
Effluent quality	High	Moderate to poor	High
Organic loading rate	Moderate	High	High
Sludge production	High	Low	Low
Footprint	High	High to moderate	Low
Biomass retention	Low to moderate	Low	Total
Nutrient requirement	High	Low	Low
Alkalinity requirement	Low	High (stream-dependent)	High to moderate
Energy requirement	High	Low	Low
Temperature sensitivity	Low	Low to moderate	Low to moderate*
Start-up time	2-4 weeks	2-4 months	Less than 2 weeks
Bioenergy recovery	No	Yes	Yes
Mode of treatment	Total	Essentially pretreatment	Total or pretreatment

*Limitations of the AnMBR technology

1.3.2. Renewable sources for bioenergy recovery

Resource recovery is the basic concept for moving towards a common scenario based on a circular economy. Biogas production through anaerobic technologies is an attractive practice suitable for this purpose. In this context Table 1.4. summarizes the biomethane potential (BMP) values associated to

different substrates that can be used for bioenergy recovery including those generated in municipalities, agricultural practices or industrial locations.

Table 1.4. Biomethanization potential values for different substrates of interest for bioenergy recovery.

	Cellulose (%)	**Hemi-cellulose (%)**	**Lignin (%)**	**C:N ratio**	**Methane potential ($dm^3 \cdot kgODS^{-1}$)***
Wheat straw	38	21	23	90	NA
Leaves	15-20	80-85	-	8-20	100-300
Rice straw	32	24	13	70	350
Primary sludge	8-15	-	24-29	-	590
Cattle manure	2-5	1-3	3-6	24	150
Municipal Solid Waste	33	9	17	40	210-220
Food waste	60	20	20	14-16	220-240
Office paper	69	12	11.3	125-850	370
Wastepaper from pulps	60-70	10-20	5-10	90	NA
Algae	20-40	20-50	-	19	90-340**

*Organic dry solids (ODS). **Data only available in $mLCH_4 \cdot gVS^{-1}$. Source: adapted from Karthikeyan and Visvanathan (2013).

Interestingly, some of the substrates summarized in Table 1.4. can be found in municipal areas, like municipal solid waste or food waste which can produce 210-220 and 220-240 $m^3CH_4 \cdot kgODS^{-1}$, respectively. Furthermore, nutrients can also be recovered from these substrates, as it is shown in Table 1.4. According to the lowest C:N ratio, feedstocks like food waste or algae contain the highest nitrogen values while also have high potential for methane production. Despite the high potential of these feedstock for bioenergy recovery, the presence of certain recalcitrant polysaccharides such as lignin, cellulose or hemicellulose difficult their use for this purpose. It should be remarked that the hydrolysis of these compounds is the first necessary step for the complete AD of the abovementioned substrates.

Some feedstocks shown in Table 1.4 are not naturally found in municipalities but are though generated in the conventional WTTPs and could be also produced in future WRRFs. Among them, the primary sludge resulting from the settler of the influent solids from sewage, presents the highest methane potential according to Karthikeyan and Visvanathan (2013): 590 $m^3CH_4 \cdot kgODS^{-1}$. Primary sludge has been traditionally used to

generate methane in side-stream anaerobic digesters of WTTPs. Algae are also shown in Table 1.4, involving all micro- and macroalgae from freshwater and marine environments. They are characterized by a wide range of methane potential (90-340 $m^3CH_4 \cdot kgODS$), depending on the microalga specie and the correspondent carbohydrates, proteins and lipids content which are detailed for some common species in Table 1.5. Both primary sludge and algae substrates are considered in the present work besides food waste, since they are generated in municipalities and/or during the waste treatment. Thus, the three of them are promising renewable sources for energy recovery according to a circular economy perspective.

Table 1.5. Several microalgae species composition and their theoretical methane potential and theoretical ammonia release during AD. Source: adapted from Sialve *et al.*, 2009.

Species	**Proteins (%)**	**Lipids (%)**	**Carbohydrates (%)**	**Methane yield***	**N-NH_3****
Euglena gracilis	39-61	14-20	14-18	530-800	54.3-84.9
Chlamydomonas reinhardtii	48	21	17	690	44.7
Chlorella pyrenoidosa	57	2	26	800	53.1
Chlorella vulgaris	51-58	14-22	12-17	630-790	47.5-54.0
Dunaliella salina	57	6	32	680	53.1
Spirulina maxima	60-71	6-7	13-16	630-740	55.9-66.1
Spirulina platensis	46-63	4-9	8-14	470-690	42.8-58.7
Scenedesmus obliquus	50-56	12-14	10-17	590-690	46.6-42.2

*Expressed in $mLCH_4 \cdot gVS^{-1}$ and **$mgN \cdot gVS^{-1}$ unit.

1.3.3. Resource recovery through implementation of microalgae technology in anaerobic-based WRRFs

Microalgae technology has been called to be the green revolution of the 21st Century. Bioengineering processes have been designed to benefit from the capacity of photosynthetic microorganisms that transform the inorganic carbon into organic molecules and energy through high harvesting. These systems therefore work as carbon dioxide sinks in which a renewable source is produced *i.e.* 1 kg of dry microalgal biomass generated can fixate 1.83 kg of CO_2. Besides, microalgae are efficient up-takers of nutrients such as

nitrogen and phosphorous as well as toxic metals from wastewaters (Sturm and Lamer, 2011). Hence, their application for resource recovery has emerged during the last decade and attracted the interest of the scientific community.

Anaerobic effluents are characterized by high contents of nitrogen and phosphorous as a result of the mineralization of the organic matter. Since these concentrations commonly exceed the legal requirements for their direct discharge to the water flows, additional nutrient removal stages are needed. In this context, microalgae technology has gaining interest, since phototrophic microorganisms can be cultivated in these effluents (Acién *et al.,* 2016; González-Camejo *et al.,* 2019). Moreover, this is very attractive from a bioenergy recovery perspective, as microalgae can be later harvested and used in AD as a substrate. This integrated process was first reported by Oswald and Golueke (1960) as a response to the first oil crisis, which triggered the need of searching alternative fuels.

1.3.4. Strategies to improve methane production from renewable sources.

Microalgae and the other feedstocks listed in Section 1.3.2 share a common drawback, which is their complex hydrolysis due to their cell-wall composition, that might result in low AD performances. The outer layers of microalgae are composed of different matrixes enriched in polysaccharides commonly including cellulose, pectin and hemicellulose (Domozych *et al.,* 2012). Moreover, highly recalcitrant compounds such as sporopollenin and algaenan can be also found among some of the microalgae that commonly grow in sewage related streams (Baudelet *et al.,* 2017). To overcome these barriers, several pre-treatment strategies of different nature have been applied to microalgae in different studies to enhance biogas production (Passos *et al.,* 2014). Notwithstanding, this have a negative impact in terms of energetic and cost demands. For this reason, several biological strategies are being explored to maximize microalgae conversion into valuable products at a low cost (Carrillo-Reyes *et al.,* 2016). A summary of all the described strategies is shown in Figure 1.4.

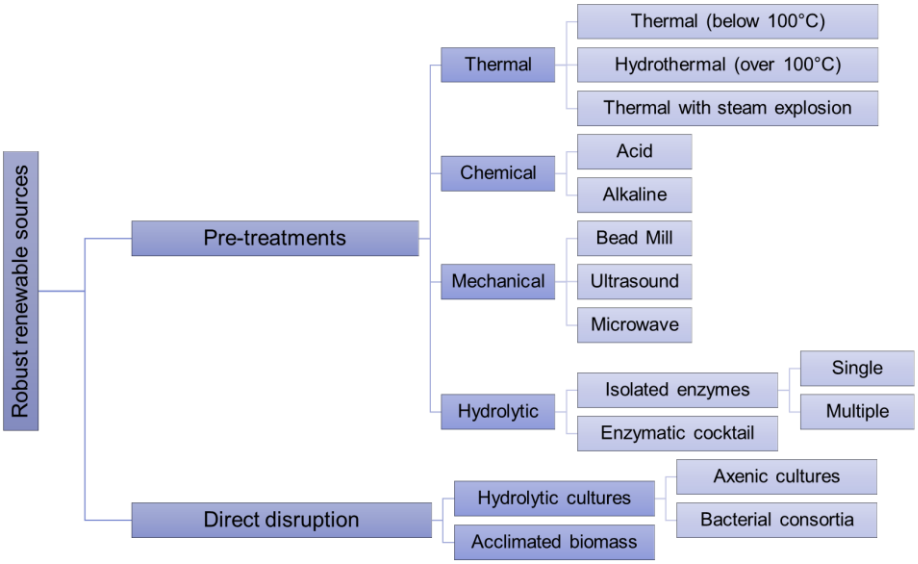

Figure 1.4. Diagram showing the different strategies to transform robust renewable sources which have low biodegradability values due to their high cellulose content (and other polysaccharides). Sources: Carrillo-Reyes *et al.* (2016); Passos *et al.* (2014).

a. Pre-treatments

Thermal, chemical, mechanical and hydrolytic strategies have been thoroughly evaluated in the literature to improve microalgal biomass conversion into biogas (Jankowska *et al.*, 2017; Passos *et al.*, 2014). Among the highest biomethanization values from microalgae was reported by Mahdy *et al.* (2014a), which achieved a production of 287 $mLCH_4 \cdot gCOD^{-1}$ after an enzymatic alkaline treatment of *Chlorella vulgaris* biomass. This pre-treatment enhanced the biodegradability of this recalcitrant microalgae to 82%. The same strategy was applied in a later study by Mahdy *et al.* (2016) over *Scenedesmus* sp., which also belongs to the phylum *Chlorophyta*, in which different microalgae typically observed in sewage-related streams are classified (also including *Chlorella* genus). The biomethanization after enzymatic alkaline pretreatment of *Scenedesmus* sp. resulted in lower values than *Chlorella,* since this microalga is the most resistant: 216 $mLCH_4 \cdot gCOD^{-1}$ were obtained, which corresponded to 62% biodegradability. Enzymatic treatments have a lower cost than other strategies based on thermal, chemical or mechanical practices. However, their application in commercial scales results challenging as a mixture of

enzymes would be needed since the composition of microalgae is heterogeneous between species. Also, a methodology for fixating the enzymes would be required in order to maintain a high hydrolysis yield (Fu *et al.,* 2010).

A promising alternative and more cost-effective strategy might be the use of microorganisms that are capable to hydrolyze the microalgae cell-wall compounds. In this manner, a continuous disruption of the biomass can be achieved as the hydrolytic microorganisms would be steadily growing and propagating as well. Several microorganisms able to disrupt robust polysaccharides and proteins have been isolated. Some of the axenic cultures have algicidal capacities, as it has been reported for *Kordia algicida*, *Alteromonas* sp., *Thalassobius aestuarii*, *Nautella* sp., *Sagittula* sp., *Thalassobius* sp., and *Pseudoalteromonas* sp. (Carrillo-Reyes *et al.,* 2016), which are classified in the *Bacteroidetes* (*K. algicida*) and *Proteobacteria* phyla (the rest of them). However, their efficiency for methane generation or microalgae continuous disruption has not been explored. Lü *et al.* (2013) did evaluate the use of *Clostridum thermocellum* to enhance in a 17-24% biomethanization of the microalgae *Chlorella vulgaris.* In a similar approach, (Yıldırım *et al.,* 2017) improved in a 18-38% the production of biogas from the marine microalgae *Haematococcus pluvialis* also using *C. thermocellum*. Moreover, some authors have benefit from both bioaugmentation and other pre-treatments such as thermal strategies. In this context, Lavrič *et al.* (2017) reported that thermal pretreatment combined with *C. thermocellum* bioaugmentation increased in a 62% the methane production but mainly due to the thermal pretreatment with steam explosion as *C. thermocellum* only improved the process in a 12%. However, the use of axenic cultures might not be viable in commercial systems or the addition of a single bacteria might not be enough to cover all the heterogeneity of the components present in recalcitrant feedstocks.

a. Direct disruption

Another alternative is the use of a combination of several axenic cultures (bacterial consortia). However, the combination of bacteria for a certain purpose might become challenging because of competition for the same

substrate. Thus, different microorganisms should be selected in order to generate a wide capacity of degradation for those compounds that provide resistance to AD feedstocks. In this context, AD processes must be carefully explored and an important effort in isolating microorganisms from specific niche should be made. Hence, the application of a bacterial consortia to enhance biomethanization of certain substrates is still on progress and would extremely depend on the availability of hydrolytic bacteria in collections of microorganisms and cell cultures. It should be remarked that this is strongly dependent to the complexity of isolating microorganisms from environmental samples.

An interesting practice to overcome the limitations of axenic cultures and hydrolytic consortia is the acclimation of the bioreactor biomass to those substrates with low biodegradability values. Most of these compounds are present in the environment and are partially or completely degraded during natural decomposition. Hence, microbial population has an inherent capacity to reduce these compounds *e.g.* plant-based components that contain lignin, cellulose among other polysaccharides. Since acclimation is possible using high retention systems, there is still a drawback in terms of time requirement, since the anaerobic growth rates are low. As an alternative, some acclimated microbial communities found in the nature such as the stomach cavity of ruminants could be used as starting inoculum of bioengineering processes for complex feedstock conversion into energy. Thus, an attractive option would be the use of the ruminal fluid of animals as an inoculum for anaerobic digesters, since ruminants can degrade some of the mentioned plant-based components due to the microbial population inhabiting their stomachs. The feasibility of the acclimation practice is thoroughly explored in the present work, which has considered the complete acclimation of the biomass as well as the use of natural hydrolytic biomass such as ruminal fluid in the optimization of systems for bioenergy production.

1.4.Microbiology in bioengineered systems

Bioengineered systems were first designed in the 1970s to replicate the conditions required for viable growth of certain microbial groups. These reactors were composed of process control systems to track environmental

parameters and keep them in the required state. In this way, the understanding and manipulation of biological systems began. Comprehension of the microbiology in bioengineered systems is crucial to their later application at commercial production scales (National Research Council Of The National Academies, 2009). A great example of how environmental conditions can be reproduced in laboratories and commercial systems are the anaerobic digesters. In these systems, the conditions required for AD of organic matter are provided to a biomass with a high microbial diversity, known as anaerobic sludge.

1.4.1. Brief history of applied microbiology

The description of the DNA structure in the 50s by Watson, Crick and Franklin lay the fundaments of the molecular biology. It was not until the invention of the polymerase chain reaction (PCR) that this area of knowledge was expanded and applied into different areas, including environmental engineering. Until that date, the study of microbiology in bioengineered systems relied on the possibility of single and pure cultivation in laboratory. However, processes such as AD result from the action of a wide diversity of microbial groups that do not behave the same way in a single pure culture and even more, might be never isolated.

1.4.2. The use of ribosomal genes as biomarkers

The culture-independent study of biological systems requires the detection of certain genes or gene fragments known as biomarkers. Ribosomes have been selected as describing elements of basic and applied life sciences and are commonly targeted using molecular biology techniques. They are key in the central dogma of molecular biology Figure 1.5, since they carry out the translation of genetic information into proteins. Besides, ribosomes are a conserved among the three domains of life *i.e. Archaea*, *Bacteria* and *Eukarya*; and can therefore provide valuable phylogenetic information.

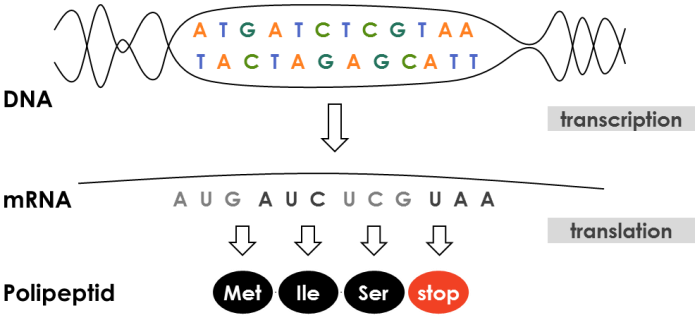

Figure 1.5. Central dogma of molecular biology.

The structure of the ribosome consists of two subunits. The small subunit of the ribosomes (SSU) has a high conservation level, but also presents high hypervariable regions concentrated in specific areas that are common for all microorganisms. For *Bacteria* and *Archaea* (prokaryotes) the ribosomal gene biomarker is known as 16S rRNA meanwhile for *Eukarya* (eukaryotes) is the 18S rRNA. The encoding sequences of these SSU are present in all members of a certain microbial group but not in the rest. Thus, they are the perfect targets to identify differences at different taxonomic levels and allow the characterization, classification and nomenclature of biological entities (Yarza *et al.*, 2014). For all these reasons ribosomes are the chosen targets in microbial diversity studies with culture-independent molecular biology techniques.

1.4.3. Conventional approaches to characterize microbial communities

The molecular biology research area has developed different techniques to characterize the diversity of different environments and niches. After the PCR development, other techniques were designed to allow the identification of different species contained in a single sample or compare the microbial diversity of different samples (Chaudhary *et al.*, 2013). Classical approaches are the terminal restriction fragment-length polymorphism (TRFLP) and denaturing gradient gel electrophoresis (DGGE). Both techniques used amplified fragments of a certain biomarker, such as the 16S rRNA gene. A region or the whole gene (1500 nucleotides) is amplified through PCR using specific oligonucleotides to a certain taxon. In TRFLP the resulting amplicons are subjected to restriction digestion by

specific enzymes, which means that the amplicons are split in particular nucleotide positions. Since this technique is only focused on the terminal fragments of the target gene, it is not powerful enough to distinguish between species that share the terminal restriction site. However, TRFLP provides information about the relative abundance of each identified group and has been successfully applied to study the dynamics, structure and diversity of a wide number of anaerobic bioreactors (Collins *et al.,* 2006). The other highlighted fingerprinting approach is the DGGE, which also uses amplified fragments of the target gene. These amplicons are denaturized with a linear gradient of formamide and urea that makes it possible to differentiate amplicons according to a small number of nucleotides. The visualization of the DGGE bands provides information about the diversity of a certain sample.

Another classical approach is cloning of a DNA fragment (*insert*) inside of another DNA molecule (*vector*) that can replicate independently from the host cell genome. This process is commonly referred as library cloning, as the result is the storage of different DNA sequences in a certain vector. For bacteria the 16S rRNA is the common targeted DNA fragment, while for archaea several studies use the mcrA gene, which encodes for the final metabolic reaction of methane production in methanogens. The main limitation of cloning is the huge effort required to obtain a representative coverage, which means that a large number of cloning libraries need to be generated in order to cover the maximum diversity. Also, this increases the cost of cloning since the last step is the read of the cloned genetic information in a sequencer. Despite these methods have been traditionally and efficiently applied to different environmental studies, their use has decayed during the last decade. Nowadays powerful techniques based on high-throughput sequencing have become more affordable and are being preferred over classical approaches due to the huge amount of information that provide.

1.4.4. Applied genomics revolution

We are living the Era of Biology. Since the publication of the human genome in the 2000s, the culture-independent methods developed are

allowing the scientific community to explore different microbial habitats such as host-associated (mainly to humans), environmental (aquatic, terrestrial and plants, among others) and also engineered systems (Figure 1.6). The last category of ecosystems includes WWTPs and bioreactors, highlighting the increasing interest of the engineering area in expanding our knowledge of the microbiology inhabiting bioengineered systems.

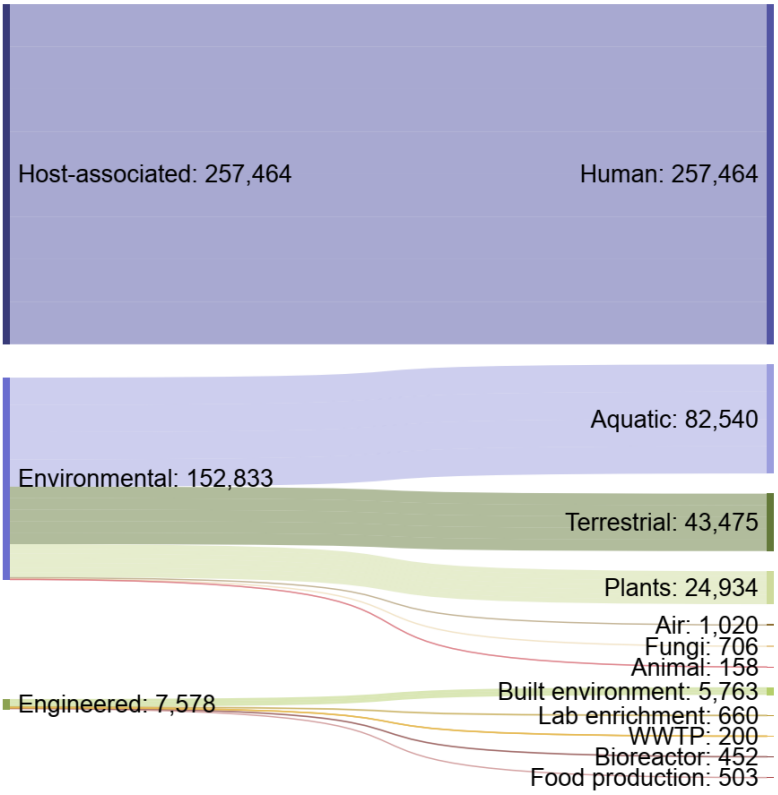

Figure 1.6. Biosample ecosystem classification available in Joint Genome Institute Integrated Microbial Genomes & Microbiomes (JG IMG) database. Downloaded May 16, 2019. The flow diagram was constructed using the online webtool *sankeymatic*. Numbers indicate the total of genome-sequencing datasets that have been deposited in the JG IMG up to date and their corresponding research area.

As Figure 1.6. shows, different sequencing projects regarding WWTP and bioreactors are being carried out worldwide, although in a much smaller number compare to other niches such as human host-associated and different environments. Precisely, two huge projects based on high-throughput sequencing have been recently published: The Human Microbiome Project

(Gilbert *et al.*, 2018) and The Earth Microbiome Project (Thompson *et al.*, 2017). Both projects pursued the definition of all the microorganisms that have colonized the different locations and how the conditions and characteristics associated to each of them have defined their microbial diversity, which refers to the term microbiome. In a similar way, the microbiome characterization of biogas producing systems is a common goal of several research groups over the world. Since AD is the result of the microbial and metabolic network established by microorganisms, its study could provide a wide comprehension of the AD process.

a. High throughput sequencing

Genome sequencing consists on the elucidation of the nucleotides and how are they sorted in a certain fragment of nucleic acids. The first methodology described was reported by Maxam and Gilbert (1977) and consisted in a chemical process that cleaves a terminally labeled DNA molecule at each position in which a base is repeated. The positions of this base are recognized through their lengths. Later, these bases are read in an electrophoresis stage, which consists on the separation of nucleic acid in an electric field according to their size and charge. The output of this technique can be visualized in an autoradiography. Over the years, DNA sequencing became less hazardous and simpler with the invention of Sanger sequencing. This method has been the most widely used one during the last 4 decades, until the apparition of massive sequencing techniques in the last decade.

The automatization of Sanger sequencing was the result of combining fluorometric markers during electrophoresis of DNA. Labeled modified di-deoxynucleotidetriphosphates (dNTPs) corresponding to each of the four standard deoxynucleotides (dATP, dGTP, dCTP and dTTP for deoxy-adenosine, guanosine, cytidine and thymidine triphosphate, correspondingly), are added during the extension of DNA in this chain termination method. The fluorescent signals are detected at the same time that the electrophoresis separates the nucleic acid fragments, resulting in a sequencing chromatogram. The main limitation of Sanger sequencing was the restriction of a single reaction. The development of high-throughput methods for nucleic acid sequencing allowed the read of different sequences

generated from more than a single sample at the same time in the same sequencing run. Indeed, these methods are known as massive sequencing for this main reason. Different companies have commercialized their sequencing techniques which could be classified according to their chemical basis in the required PCR previous reaction to sequencing (emulsion or bridge PCR) and during nucleotide reading (pyrosequencing, ligation or synthesis). Among the different technologies, the one developed by Illumina (San Diego, USA), based on PCR bridge amplification and sequencing by-synthesis has been preferred over the rest of them.

a. Basis of Illumina sequencing technology

The concept of Illumina technology is similar to Sanger capillary electrophoresis-based sequencing. In this approach, a DNA polymerase catalyzes the introduction of dNTPs into a DNA template strand during several sequential cycles of DNA synthesis. These dNTPs are recognized through fluorophore excitation and detection. The main difference with previous sequencing strategies is that in Illumina sequencers, millions of fragments are amplified simultaneously revealing their nucleotide composition or sequence in a massive parallel stage. A conventional Illumina workflow for high-throughput sequencing (Figure 1.7) consists on: (i) library preparation, (ii) cluster generation, (iii) sequencing and (iv) data analysis. The first stage refers to the amplification with barcoded primers of the target gene using DNA or complementary DNA (cDNA). These special primers are oligonucleotides containing the specific region to amplify the desired gene and different fragments of nucleotides named barcodes, which are composed of an index and an adapter. The indexes are 8-12 base pairs (bp) long unique combinations that are paired to each sample. This allows the later identification of different samples being sequenced in during the same sequencing run. Besides the barcode, each primer contains a 20-30 nucleotide length adapter that allows the fixation of the nucleic acid fragment to the Illumina sequencing flow cell. These adapters are complementary to the lawn of oligos present in the flow cell.

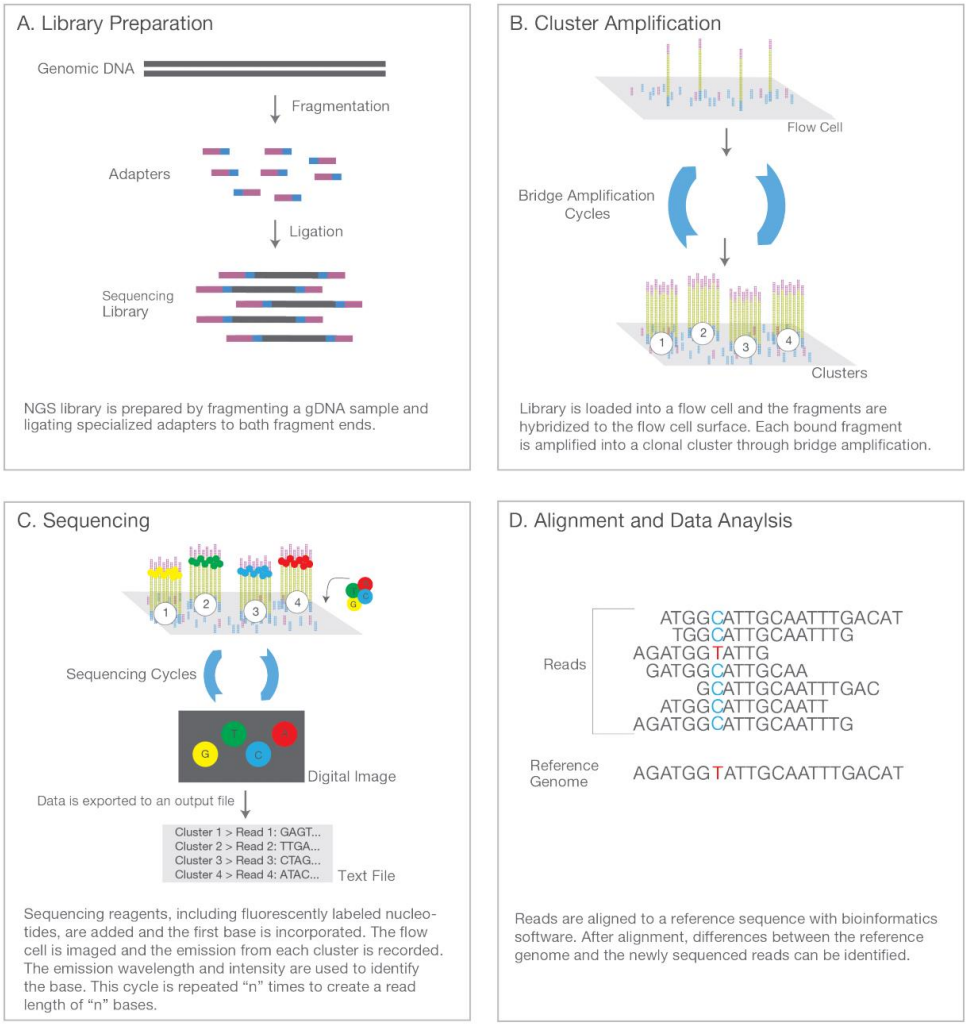

Figure 1.7. Conventional Illumina workflow for high-throughput sequencing. Source: Illumina Inc. (2017).

The second stage consists on the cluster generation through continuous attachment of the previously synthetized DNA fragments (Illumina libraries) to the sequencing flow cell. After this stage, each fragment is amplified into distinct clonal clusters through bridge amplification (see Figure 1.7b). The third stage starts with the synthesis and simultaneous detection of the nucleotides being incorporated during clonal amplification (Figure 1.7c). During this step digital images are taken reflexing the colors identified,

which are specific to each nucleotide that has been incorporated to the synthetized DNA during the sequencing run. The Illumina sequencing output is a high accurate database with low biases and error rates. The fourth and final stage of the Illumina workflow is the data analysis, which combines several bioinformatics steps until the raw sequences with low quality are discarded, the barcodes are removed, and the samples are demultiplexed revealing the sequences obtained for each sample. Moreover, in a paired-end bridge amplification strategy, sequences resulting from the first and second read are merged in a single one. The Illumina paired-end approach was an important innovation, since it enhanced the capacity of this sequencing strategy to read longer regions of the target genes raising the coverage up to 600 bp.

b. Bioinformatic pipelines for sequencing data downstream processing

The huge amount of data produced by next-generation sequencing requires an intense computational downstream processing. Regarding microbial ecology and phylogenetic studies, different bioinformatic pipelines have been developed during the last decade. Among them, two open source software should be highlighted since they are the most used in the reported literature. One of them was developed by Caporaso *et al.*, (2010) and it is known as quantitative insights of microbial ecology (QIIME). The other was created by Schloss *et al.* (2009) and it is known as Mothur. Both QIIME and Mothur pipelines allow the filtering of the raw 16S rRNA gene sequences retrieved from Illumina and other sequencing platforms. The filtering process includes the removal of homopolymers and chimeras. The term homopolymer refers to the strings of repetitive nucleotides that can be formed during amplification processes. The second concept, chimera, refers to the DNA artifacts that might be formed during sequencing process. They are considered contaminants since they can be wrongly interpreted as novel sequences (Schloss *et al.,* 2011). After obtaining a clean sequence of the target gene (commonly 16S rRNA gene for bacteria and archaea profiling studies), the dimensions of the data are subsequently reduced using clustering techniques. Filtered and clean datasets are free of homopolymers, chimeras, and replicated sequences belonging to the same microorganism. Traditionally, phylogenetic studies

have searched the aggrupation of different sequences at the lowest taxonomic level possible *i.e.* species. It is known that similarity percentages between sequences over the 80% define the genus level. For the species, it can be considered that a dissimilarity below 3% between two certain sequences assigns them into two different species. Some studies apply a more restrictive dissimilarity percentage of 1%. Resulting clusters at a certain dissimilarity level are referred as operational taxonomic units (OTU) in microbiological studies.

In Mothur and QIIME pipelines different algorithms for clustering generation according to a dissimilarity percentage are included. Several OTU clustering strategies known as "OTU picking" can be found: (i) closed reference, (ii) open-reference and (iii) de novo reference. The first of them only considers those sequences contained in a reliable dataset that has no chimeric sequences (a single sequence originated from multiple transcripts) of a certain target gene. The second strategy does not use any dataset and therefore clusters all the sequences regardless of their existence in a nucleotide collection. The third OTU picking strategy first clusters the sequences against a known gene reference database, such as the closed reference strategy. Later, those sequences that have not been assigned to any known OTU are further clustered generating new reference OTU that can be interpreted as different species with the identified OTU in microbial ecology analysis. Finally, OTU are classified according to a database that contains the target gene sequences of several bacteria or archaea microorganisms. Two of the most common are the ribosomal database project (RDP) database (Cole *et al.*, 2014) and SILVA (Pruesse *et al.*, 2007; Quast *et al.*, 2013), that have been constructed according to the work of Goodfellow *et al.* (2012), developers of the Bergey's Manual for taxonomy. The better the underlying database, the better the classification of sequences. Since no consensus in the microbiology for OTU assignment has been proposed and the different combination of algorithms and sequence downstream processing methods have their own intrinsic and unavoidable biases, the selection of a pipeline for microbial ecology analysis is crucial to guarantee the later interpretation of data and comparison between different studies (Hugerth and Andersson, 2017).

1.4.5. Biostatistics to link microbial ecology and bioengineering factors

Microbial community composition from a certain environment depends on its abiotic and biotic parameters. In an AD context, the abiotic factors are the operational conditions set in the bioreactor, such as temperature or others related to the bioreactor configuration (SRT, HRT, coupling of membranes, carriers, among others). These parameters are also known as deterministic factors, a concept that also includes biotic aspects such as interspecies interactions and substrate availability. As demonstrated by Vanwonterghem *et al.* (2014a), deterministic factors shape microbial communities in anaerobic digesters. Thus, the study of the link between both microbial community and deterministic factors is a necessary step in current AD studies, especially when treating complex substrates that might need more specific and subtle conditions to achieve a higher hydrolytic potential of the microbial population (Shrestha *et al.*, 2017).

The generation of thousands of sequences from different species inhabiting anaerobic digesters allows the study of how the operational conditions set in AD shape microbial community structures. Moreover, the co-existence of certain groups in a same niche also provides important information, since AD is the result of a complex network of microbial interactions and metabolic pathways (Figure 1.2). In this context, biostatistics have gained noticeably relevance during the last decade in applied microbiology studies in AD systems. Since the datasets generated by high-throughput sequencing combined with the performance data of the bioreactors result in a complicated data analysis challenge, several multivariate techniques are needed to achieve a complete comprehension of the microbial ecology behind a certain AD process (Hugerth and Andersson, 2017; Ramette, 2007).

a. Multivariate analysis

Figure 1.8 summarizes some of the most common multivariate analysis techniques and those that have been used in the present work. According to Ramette (2017) on microbial ecology studies the most common analysis is based on exploratory approaches such as principal component analysis (PCA) or clustering methods. The exploratory techniques can be subdivided

in unconstrained ordination methods or clustering. The first category comprehends gradient analysis techniques such as multidimensional scaling (MDS), non-metric MDS (NMDS), principal component analysis (PCA) and principal co-ordinate analysis (PCoA). The three of them share a common aim, which consists on finding the explanation of the variability in between samples in terms of species composition (univariate response) because of environmental variables. In the case of AD, these explanatory variables can be qualitative or quantitative, such as the inoculum source or the operational conditions of the reactor among others, respectively. The ordination methods are more constraint when the number of exploratory variables is low and thus, they directly examine the relationships between different sets of variables (direct gradient analysis). However, when the number of environmental variables is greater than the number of samples minus two, the ordination is considered unconstrained (indirect gradient analysis). This is a typical scenario in AD microbial ecology studies. Finally, the ordination methods for gradient analysis are also available in their partial version. Whereas in the original method the statistical indicator used to ordinate samples is the variance, in partial ordination the variability among samples is explained through the maximization of the covariance.

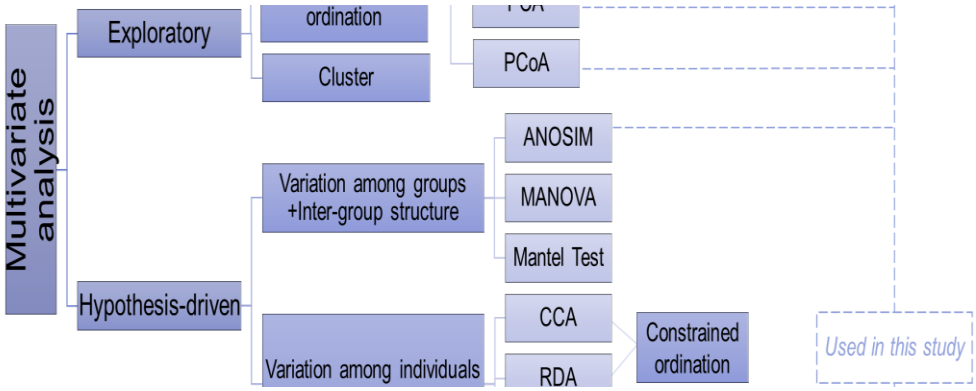

Figure 1.8. Multivariate analysis techniques. Abbreviations stand for: multidimensional scaling (MDS), principal component analysis (PCA), principal co-ordinate analysis (PCoA), analysis of similarities (ANOSIM), multivariate analysis of variance (MANOVA), canonical correspondence analysis (CCA), redundancy analysis (RDA), partial least square (PLS) and partial least square discrimination analysis (PLS-DA). Source: adapted from Ramette (2007).

a. Exploratory approaches and ordination methods

The common goal of ordination methods such as MDS, PCA or PCoA is to find a projection on a multidimensional space with reduced dimensions (two or three are preferably used) through latent variables generation. These variables are the ordination axes and allow the separation of samples according to their differences and similarities. The output is plotted in the corresponding dimensions as Figures 1.9a and 1.9b show, allowing the interpretation and elucidation of gradients between samples. In AD systems, the species composition per sample dataset is commonly used as the main matrix to compare the ecological status of the reactors. While PCA requires the use of Euclidean distances matrix to compare the different samples, MDS does not require a specific data matrix and can instead use distance matrixes based on ecological estimators such as Bray-Curtis or the UniFrac distances.

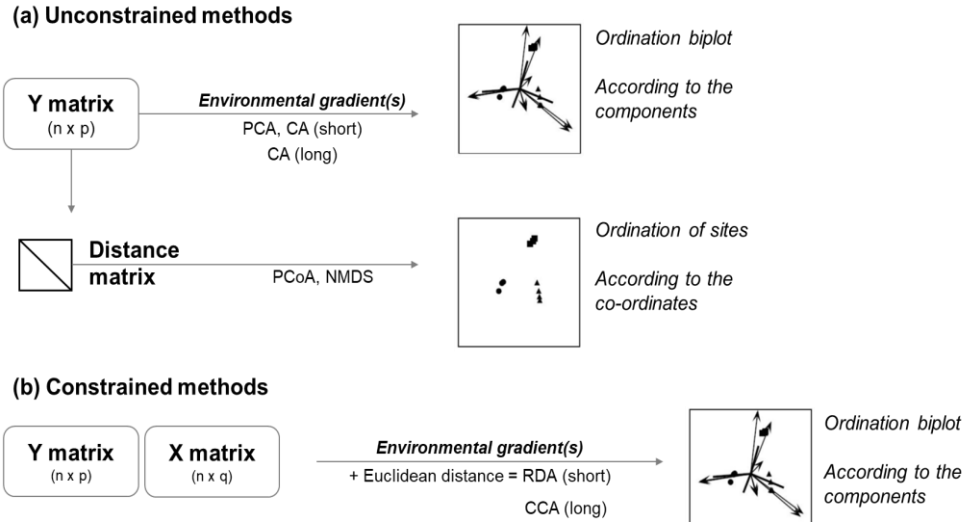

Figure 1.9. Unconstrained (a) and constrained ordination methods used for multivariate statistical analysis. Abbreviations are environment or sample analyzed (n), species or exploratory variables (p) and environmental or performance explanatory variables (q), principal component analysis (PCA), canonical analysis (CA), principal co-ordinate analysis (PCoA), non-metric multidimensional analysis (NMDS), redundancy analysis (RDA) and canonical component analysis (CCA). Source: adapted from Legendre and Legendre (2012).

The Bray-Curtis method (Bray and Curtis, 1957) relies on the dissimilarity in between samples to calculate a distance matrix. The counts on each site are used as the inputs as Equation 1 shows. In this equation, samples *i* and *j* are analyzed in terms of species composition and the distance between the two communities is indicated as the Bray-Curtis estimator (BC_{ij}). This estimator results from the sum of the smaller distance values of the shared species between samples *i* and *j*. S_i and S_j are the total amount of species identified in all samples.

$$BCij = 1 - \frac{C_{ij}}{S_i + S_j} \text{ (Equation 1)}$$

The UniFrac distance (Lozupone and Knight, 2005) improves the ecological analysis of dissimilarities, since it includes phylogenetic information that allow the exploitation of the divergence degree between different sequences extracted from an environmental microbial sample analyzed through massive sequencing targeting biomarkers (such as the 16S rRNA gene). Besides ordination techniques, the clustering methods provide valuable information and an easy interpretation of the similarities between samples. Hence, the cluster is a classification method that searches the generation of groups with internal homogeneity in terms of samples and species. Among the different types of clustering techniques, one of the most common in microbial ecology studies is the non-hierarchical clustering which is also called *k*-means clustering. The procedure consists on the minimization of the within-cluster variability and maximization of the between-cluster variability. The term *k* refers to the number of desired clusters fixed before the analysis. The output of clustering is a tree or dendrogram that visualizes the differences and similarities between samples.

b. Hypothesis-driven approaches

After the exploratory analysis and gradient detection, hypothesis about the environmental variables of AD systems that are modifying their microbial diversity can be formulated. These hypotheses can be tested to find the significance of the variation among groups and the inter-group structure. Examples of methods for this purpose are summarized in Figure 1.8, comprehending analysis of similarities (ANOSIM), multivariate

analysis of variance (MANOVA) or the Mantel test. On the other hand, the significance of the variation among individuals and the environmental gradients detected can be elucidated using canonical correspondence analysis (CCA), redundancy analysis (RDA) and partial-least square analysis (PLS). From all these methods, in the present study the ANOSIM and the PLS methods have been used jointly with the previously explained exploratory methods to elucidate the relationship between the operational conditions of the reactor (using both quantitative and qualitative variables) and the microbial community structures.

The PLS method (Wold, 1966; Wold *et al.*, 2001) allows the modelling (in regression or canonical form) of two common datasets retrieved from microbial ecology of AD studies such as the species composition (Y matrix) and the environmental variables (X matrix) (Figures 1.10a, 1.10b). An interesting advantage of this technique compared to other multivariate analysis is the inclusion of correlated variables, since these variables are discarded in traditional multiple regression models. From the point of view for future application of microbial ecology analysis into bioengineering processes, the elucidation of a model of response of certain microorganisms to environmental variables could report valuable information. The principle of PLS consists on the maximization of the covariance between two datasets by seeking linear combinations of the variables retrieved from both sets which are referred as latent variables. In this analysis, loading vectors are generated as a result of the linear combinations observed as Figure 1.10a shows. It should be highlighted that PLS can be also used as a classification method in its discriminant analysis variant (PLS-DA). In this case, the first matrix composed of the species diversity is related to a class vector or factor such as the inoculum source or the reactor configuration in AD studies; that indicates the class of each sample (Figure 1.10b). Hence, the PLS-DA allows to explore the influence of a qualitative variable over the microbial structure of AD systems. This is an important step during process optimization in complex studies in which microbial responses are key to guarantee the good performance of the bioreactors. For this reason, PLS-DA has also been used in the present study to select the operational parameters that most influence the microbial structures observed in different AD systems.

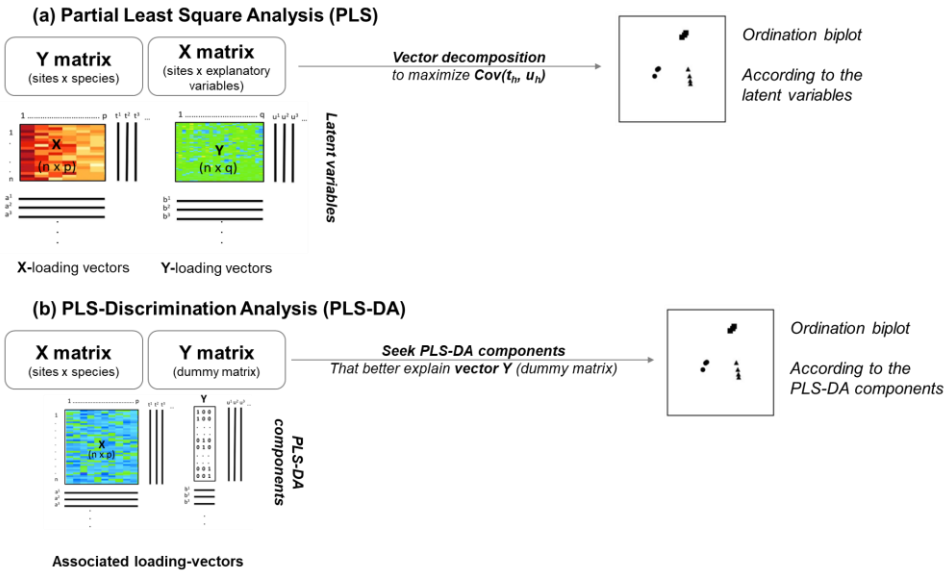

Figure 1.10. Explanation of the Partial Least Square (PLS) and PLS-Discriminant Analysis (PLS-DA) methods. (a) PLS matrix decomposition into sets of latent variables and loading vectors and (b) the PLS-DA matrix decomposition into sets of PLS-DA components (latent variables) and loading vectors. Abbreviations are environment or sample analyzed (n), species or exploratory variables (p) and environmental or performance explanatory variables (q). Source: adapted from González *et al.* (2013).

Finally, another hypothesis-driven method shown in Figure 1.9 and used in the present work is the ANOSIM test. This technique is commonly used in microbial ecology to test spatial differences, temporal changes or environmental impacts on microbial structures (Clarke, 1993). The aim of ANOSIM is to test for significant differences according to distance measurements between different groups of samples (clusters) (Clarke, 1993). The output is the R statistic measure (R_{ANOSIM}) that corroborates separation between clusters (R_{ANOSIM} =1) or not (R_{ANOSIM} =0). ANOSIM is commonly combined with the previously mentioned ordination methods and is especially useful to determine the significance of the clusters of samples observed in PCA, PCoA, and MDS after ordination according to the components retrieved.

1.5. Current status of biogas microbiome characterization and microbial ecology patterns in anaerobic digesters

The term black-box has been traditionally assigned to anaerobic digesters because of the lack of knowledge of the microbiological processes that are carried out during AD by different microorganisms. In this context, the knowledge had been limited by the technology available to enlighten the different microbial groups involved in the complex network of AD. Nevertheless, the last decade advances in sequencing technologies (Section 1.4.4) has expanded the possibilities to analyze microbial communities, including the complex networks inhabiting anaerobic digesters.

Changes in the microbiome structure of anaerobic digesters can potentially be interpreted and used as (in)stability indicators of this bioenergy production process (Koch *et al.*, 2014). Since interspecies relationships and synergic metabolic pathways influence AD, the study of single species cannot provide enough information about the health status of anaerobic digesters. Hence, the application of high-throughput sequencing techniques that allow the characterization of AD microbiomes is a valuable tool for the management of bioengineering systems. In this context, several studies can be found in the literature, seeking the definition of an AD microbiome (Rivière *et al.*, 2009). Related to this, Figure 1.11 shows a pipeline to analyze the link between performance and microbial ecology of a certain bioengineered system.

The different technologies that allow the detection and quantification of cellular molecules such as genes, transcripts, proteins and small metabolites are known as omics. The present work has been mainly developed using a genomics through 16S rRNA gene amplicon sequencing, except for the last chapter that applied a transcriptomic approach (targeting the 16S rRNA). Several authors have highlighted that the merging of the data collected using different omics in a single database would be a required step for future management of bioengineered systems (Vanwonterghem *et al.*, 2014b). Nevertheless, all these methods are costly, technologically complex and demand specialized equipment and personnel. Furthermore, according to the current situation there is a remarkable holding time between the data

acquisition and its final interpretation. Nevertheless, automation of the methodology is possible and allows the reduction of the hands-on sample preparation time as well as the biases introduced by human manipulation of the samples.

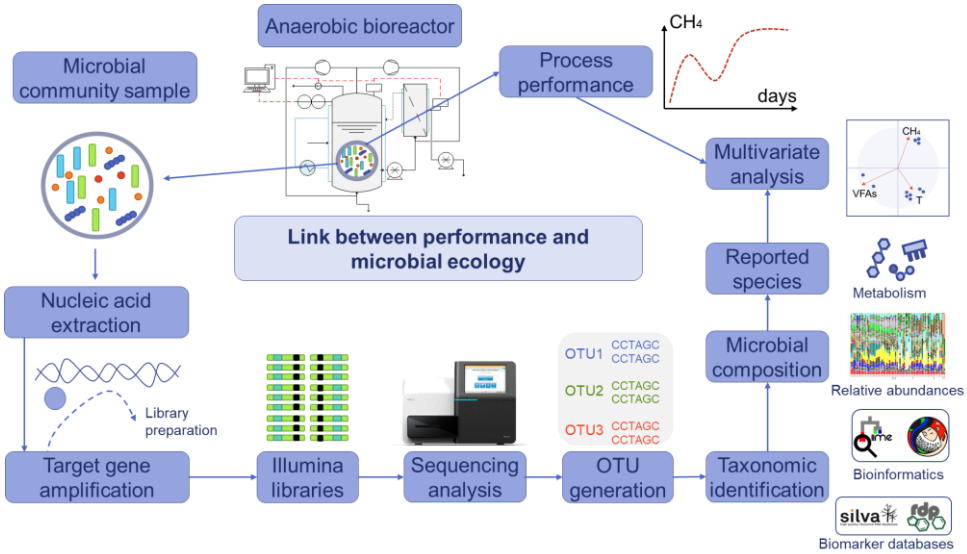

Figure 1.11. Scheme for 16S rRNA gene interpretation of sequencing data and microbial ecology.

Although further development of automatized technologies for nucleic acid extraction (or other target compounds) and their sequencing preparation is still needed, currently available equipment allows the analysis of hundreds of samples in a short time. A good example of integration of metaproteomics, metatranscriptomics and metaomics was reported by Hassa *et al.* (2018) who summarized the archaeal and bacterial diversity of 78 full-scale anaerobic digesters belonging to different studies published between 2008-2017. This study includes both mesophilic and thermophilic full-scale biogas plants and provides an integrated vision of the bacterial and archaeal diversity in these systems (Figure 1.12). Focusing on the genomics, the study reported lower abundance groups under mesophilic than under thermophilic conditions.

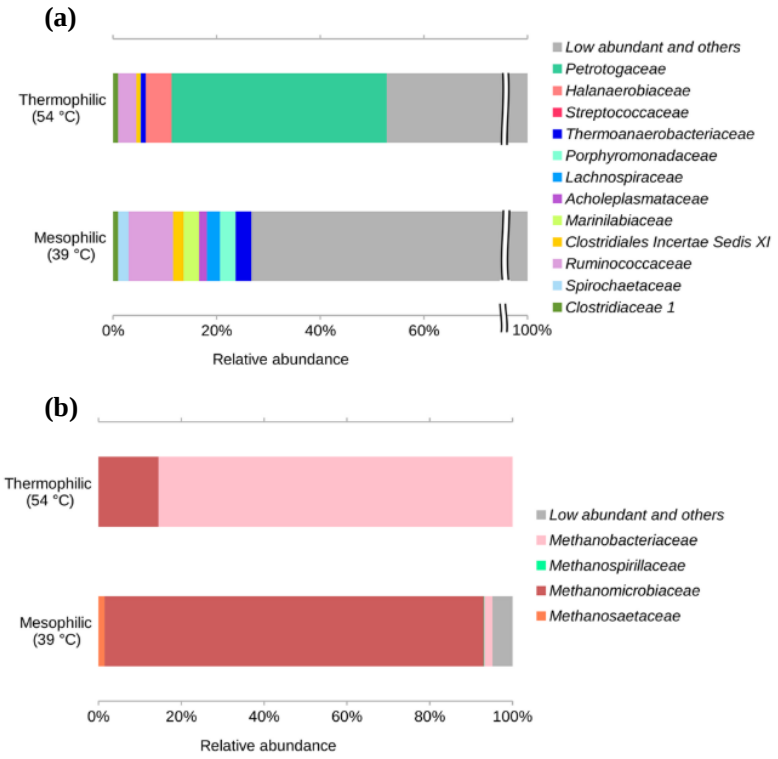

Figure 1.12. Barplots show the relative abundance of dominant (a) *Bacteria* and (b) *Archaea* families in typical thermophilic and mesophilic biogas plants. Source: Hassa *et al.* (2018).

Another good example of the potentials of these techniques can be found in Campanaro *et al.* (2016). The study revealed the relationship through metagenomic studies of the different microorganisms and their specific metabolic stages during AD (Figure 1.13). Microorganisms are plotted in the same color when belonging to the same phylum. As it can be seen, *Firmicutes* (Fi-, red) and *Proteobacteria* (Pr-, blue) are the most dominant groups and especially the first of them, is present in almost all the stages regardless of the methanogenesis, which is specific to the *Euryarchaeota* phylum (Eu-, pink). In the hydrolysis stage, the role of *Spirochaeta* was highlighted (Sp-, yellow), as some *Synergistes* (Sy-, purple). Furthermore, the authors observed a remarkable implication of *Bacteroidetes* (Ba-, green) in polysaccharides utilization. The metabolism related to the cycle of sulfur

gathers different taxonomic groups: *Proteobacteria*, *Firmicutes*, *Euryarchaeota* and *Synergistes* microorganisms are able to use inorganic and sulfur forms as electron acceptors. On the contrary, steps related to the nitrogen cycle such as ammonia conversion into nitrogen are more specific to certain *Proteobacteria* and *Bacteroidetes* groups.

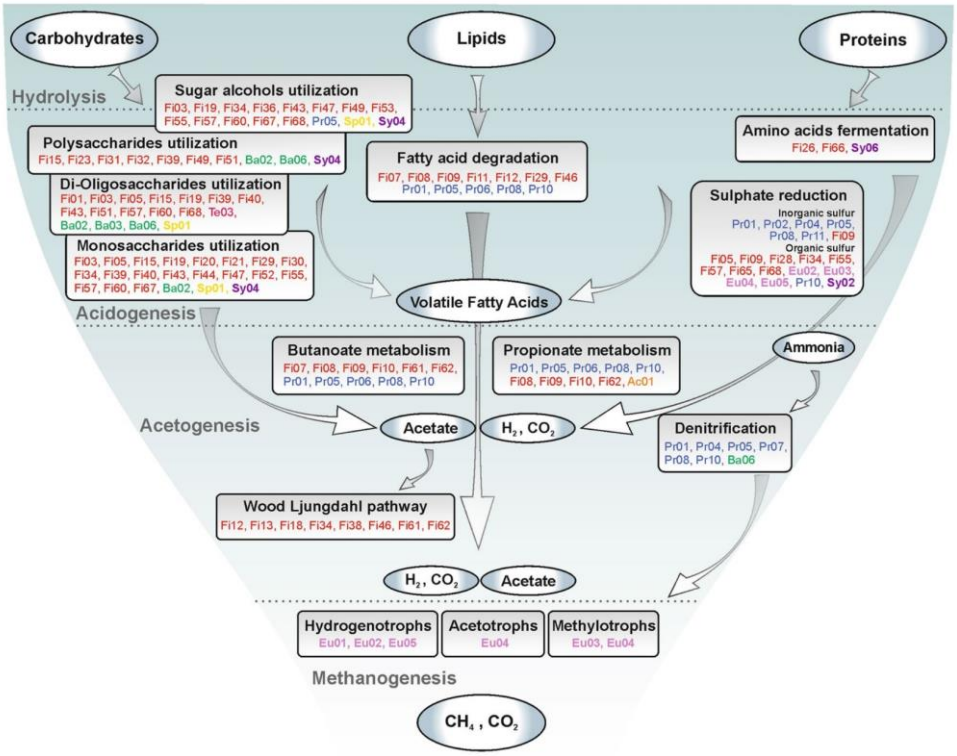

Figure 1.13. Metagenomic expanded diagram of organic matter AD. Source: Campanaro *et al.* (2016).

Hence, the traditional diagram of AD is being expanded nowadays with the current omics approaches and in an increasing trend. As several studies have been published over the last decade targeting sludge samples of different biogas producing systems, the diversity inhabiting these bioreactors has finally started to be well known. However, there are pattern differences depending on the substrate that is mainly treated and the performance of the bioreactor itself and the response in case of a failure. Related to this, different studies exploring AD of renewable sources with

high energy content (such as microalgae, lignocellulosic substrates, food waste and different sources of sludge) have been summarized in Table 1.6. As it can be seen in this table, some of them have been performed in batch experiments in small volume reactors up to 1 L, except for Barragán-Trinidad *et al.* (2017) who performed *Scenedesmus* AD with rumen in 4 L reactors. Substrates different than microalgae have been explored in higher volume systems, such as agricultural waste that was co-digested in 8 L batch reactors (Liu *et al.,* 2017). Besides, some continuous systems using CSTR or Anaerobic Sequencing Batch Reactor (ASBR) bioreactor configurations have been also reported. However, most of these studies have been developed under constant operational conditions. Regarding the sequencing approach applied in each study, it can be seen a global trend in the use of sequencing by synthesis (Illumina, USA). As pointed out in the Section 1.4.4, the balance between cost and sequencing depth motivated its application during the last decade over other sequencing technologies such as pyrosequencing (Barragán-Trinidad *et al.,* 2017; Sanz *et al.,* 2017; Sun *et al.,* 2015). According to the different primer combinations chosen, the 16S rRNA gene is the most targeted gene through the 341F-805R (Klindworth *et al.,* 2013) or 515F-805R (Caporaso *et al.,* 2011) primer pairs for the hyper-variable regions of the 16S rRNA gene v3 to v4 and v4, respectively. Moreover, Archaea and Bacteria are commonly targeted in a simultaneous sequencing approach although a parallel detection using different primer pairs (like in the study of Koo *et al.* (2019)) can provide a better caption of the Archaea diversity. However, the detection in separate of both domains has a higher cost and thus, prokaryotic primers targeting both groups of microorganisms is commonly preferred. Interestingly, some studies included the study of Fungi eukaryotic kingdom through quantitative PCR (Aydin *et al.,* 2017) or amplicon sequencing (Fisgativa *et al.,* 2017) since they have a potential implication in the hydrolysis stage of complex substrates such as lignin (Kazda *et al.,* 2014; Shrestha *et al.,* 2017).

The identification of the *Archaea* diversity by the studies summarized in Table 1.6 is consistent with the results from Hassa *et al.* 2018 (Figure 1.12). Regarding *Bacteria*, most of the phyla are observed in all studies regardless of the source of feedstock and the choice of primers such as *Firmicutes* or

Bacteroidetes. Moreover, as mentioned in Section 1.4.4, there is not a consensus in the bioinformatics pipeline used among studies but Mothur or QIIME are the most reported tools (Table 1.6). Finally, different biostatistics analysis are reported in the studies referenced in Table 1.6 being the exploratory multivariable analysis the preferred options (Figure 1.8) including cluster, PCoA, PCA and NMDS. The lack of statistical analysis is common related in these studies to the experimental design since the bioreactors have been evaluated under constant operational conditions.

Although all the studies include interesting information about the microbial community structures, not all of them include information about the response of the population to the changes in the deterministic factors *i.e.* operational parameters of the digesters. Since specific communities might be required to enhance a direct disruption of recalcitrant substrates for biogas production, the study of the influence of certain parameters (temperature, HRT or SRT, among others) should be carefully explored. The present work mainly focuses on this aspect and seeks the definition of diverse core microbiomes for each process of bioenergy production from renewable sources. Furthermore, the AnMBR technology has been used in the present work to enhance the biodiversity of the systems through operation at high SRT (over 50 days) while maintaining low HRT. This aspect is a remarkable difference to the current studies reported about bioenergy generation, since only a few AnMBR have been applied to this purpose and most of the studies lack of microbial information.

Table 1.6. Review on renewable sources for bioenergy producing studies with focus on microalgae and food waste feedstocks.

Reference	Feedstock	T (ºC)	Reactor, pretreatments	Methane yield	Sequencing	Bioinf.	Microbial core	Biostatistics
Aydin 2016	*Haematococcus pluvialis*	55	1.5 L ASBR. Bioaugmentation with hydrolytic bacteria (*C.thermocellum*)	499 $mLCH_4 \cdot gVS^{-1}$	Illumina, 16S rDNA, 518F-926R, 518F-958R	n.d.	*Archaea: Methanosaeta, Methanosarcinales Bacteria: Firmicutes, Bacteroidetes*	ANOVA, Pearson correlation tests
Aydin 2017	*Haematococcus pluvialis*	41	1.5 L ASBR. Bioaugment. with rumen of granular sludge	600 $mLCH_4 \cdot gVS^{-1}$	Illumina, 16S rDNA, 518F-926R, 518F-958R qPCR for Fungi	n.d.	*Archaea: Methanosaeta, Methanomethylovorans, Methanobacterium, Methanosphaerula, Methanosarcina and Methanolinea. Bacteria: Synergistetes, Planctomycetes, Chloroflexi, Lentisohaerae, Thermotogae, Verrucomicrobia, Firmicutes Proteobacteria, , Actinobacteria, Chloroflexi.*	ANOVA, Pearson correlation tests
Barragán-Trinidad et al., 2017	*Scenedesmus* sp.	40	4 L batch tests. Use of hydrolytic rumen culture as inoculum	193 $mLCH_4 \cdot gCOD_{inf}^{-1}$	Pyroseq., 16S rDNA, 28F-338R	QIIME	*Bacteria: Bacteroides, Prevotella and Paludibacter*	Cluster
Cho et al 2018	*Cyanobacteria* from a river bloom	35	1 L anaerobic fermenters. Alkaline and thermal-alkaline pretreatments	Fermentation study	Illumina, 16S rDNA, 341F-805R	Mothur, SILVA database	*Firmicutes, Bacteroidetes, Chloroflexi and Proteobacteria*	PCoA
Deng et al., 2018	Lignocellulosic (corn stalks, guinea grass, rice straw)	39	2.5 L CSTR. Use of hydrolytic rumen culture as inoculum	220 $mLCH_4 \cdot gCOD_{inf}^{-1}$	Illumina, 16S rDNA, 341F-806R	QIIME	*Archaea: Methanobacteriales Bacteria: Bacteroidetes, Firmicutes (Pelotomaculum and Syntrophobacter), Spirochaetes, Synergistetes, Proteobateria*	Alpha diversity estimators, PCA

(Continues in next page)

Reference	Feedstock	T (ºC)	Reactor, pretreatments	Methane yield	Sequencing	Bioinf.	Microbial core	Biostatistics
Di Maria and Barrata, 2015	Sludge and fruit vegetable waste co-digestion	35	100 L CSTR. No pretreatment	435 $mLCH_4 \cdot gVS^{-1}$	Illumina, 16S rDNA, 515F-806-R	QIIME	*Archaea: Methanosaeta* *Bacteria: Gammaproteobacteria, Chloroflexi (Treponema), Spirochaetes, Bacteroidetes and Firmicutes*	no
Fisgativa et al., 2017	Food waste	38	520 mL batch tests. No pretreatment	420.8 $mLCH_4 \cdot gVS^{-1}$	Illumina, 16S rDNA, 515F-928R, 18S rDNA 0817F-1196R	QIIME	*Bacteria: Fimicutes,Bacteroidetes, Proteobacteria* *Fungi: Eurotiomycetes,Saccharomycetes and Basidiomycota*	Pearson correlation test
Klassen et al., 2017	*C. reinhardtii*	38	1 L CSTR. No pretreatments	416-462 $mLCH_4 \cdot gVS^{-1}$	Illumina, 16S rDNA, 341F-806R	UPARSE, RDP Classifier	*Bacteria: Bacteroidetes, Firmicutes, Thermotogae*	no
Koo et al., 2019	Food waste	35	Full scale digesters	n.d.	Ion torrent 16S rDNA, 518F-805R, 787F-Arc1059R	UPARSE, Silva database	*Archaea: Methanobacterium, Methanobacterium, Methanoculleus, Methanoculleus and Methanobacterium* *Bacteria: Acholeplasma, Caldicoprobacter, Mobilitalea, Petrimonas, Rikenellaceae, RC9 gut group, Sphaerochaeta Fastidiosipila, Lutispora, Alkaliphilus, Syntrophomonas, Clostridium, Gelria, Tissierella, Treponema, Ercella, Dethiobacter, Sedimentibacter, Lactobacillus, Proteiniphilum, Cryptanaerobacter and Papillibacter*	Alpha diversity estimators, NMDS, RDA, PERMANOVA and ANOSIM-R
Li et al., 2017	*Chlorella* sp. co-digestion with chicken manure	35	200 mL batch tests. No pretreatment	238.7 $mLCH_4 \cdot gVS^{-1}$	Illumina, 16S rDNA, 338F-806-R, 524F-Arch958R		*Archaea: Methanosaeta and Methanosarcina* *Bacteria: Bacteroidetes, Proteobacteria, Firmicutes and Spirochaetae*	no

(Continues in next page)

Reference	Feedstock	T (ºC)	Reactor, pretreatments	Methane yield	Sequencing	Bioinf.	Microbial core	Biostatistics
Liu et al., 2017	Mixed sludge, thin stillage, agricultural waste	37	8 L batch tests. No pretreatment	261-390 $mLCH_4 \cdot gVS^{-1}$	Illumina, 16S rDNA, 515F-805R	QIIME	*Archaea: Methanobacterium, Methanobrevibacter Methanosarcina and Methanobacteriales. Bacteria: Bacteroidetes and Fimicutes*	PCoA
Sanz et al 2017	Chlorella vulgaris	35	1.5 L CSTR. No pretreatment	85 $mLCH_4 \cdot gCOD_{inf}^{-1}$	Pyroseq., 16S rDNA, 27F - 907R	Mothur, SILVA database	*Proteobacteria, Bacteroidetes, Chloroflexi, Firmicutes*	no
Sanz et al 2017	Chlorella vulgaris	35	1.5 L CSTR. Thermal pretreatment	126 $mLCH_4 \cdot gCOD_{inf}^{-1}$	Pyroseq., 16S rDNA, 27F - 907R	Mothur, SILVA database	*Spirochaetae and Actinobacteria*	no
Sun et al., 2015	Straw and cow manure	37, 44, 52	5L CSTR. Steam explosion No pretreatment	100 $mLCH_4 \cdot gVS^{-1}$	Pyroseq., 16S rDNA, 8F - 515R, 340F-1000R	QIIME	*Bacteria: Firmicutes, Bacteroidetes and Cloacimonetes Archaea: Methanosarcina, Methanoculleus and Methanobacterium*	PCoA,
Yi et al., 2014	Food waste	35	6 L CSTR No pretreatment	700-870 $mLCH_4 \cdot gVS^{-1}$	Pyroseq., 16S rDNA, 27F-533R, 344F-915R	Mothur, SILVA database	*Chloroflexi and Methanosarcina besides Bacteroidetes and Firmicutes*	no

Abbreviations: Continuous Stirred Tank Reactor (CSTR), Anaerobic Sequencing Batch Reactor (ASBR), Analysis of Variance (ANOVA), Principal Co-ordinates Analysis (PCoA), Principal Component Analysis (PCA), Non-metric Multidimensional Scaling (NMDS), Redundancy Analysis (RDA), Permutational Multivariate Analysis (PERMANOVA), Analysis of Similarities (ANOSIM).

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2. Aims and Objectives

2. Aims and Objectives

This research work aimed at deepening the understanding of the microbial ecology of anaerobic digestion processes for bioenergy production from renewable sources that are generated in municipalities or that result from the treatment of sewage. The influence of the operational parameters used in bioengineered systems at both pilot- and laboratory scales has been explored in this work.

The global objective of this work was the elucidation of the structure and dynamics of key microbial groups involved in bioenergy producing processes and that can be integrated into the treatment of urban wastewater on a circular economy frame. The project key objectives were to:

- Study the enhancement of the hydrolytic potential of microbial populations in different bioreactor configurations at both the laboratory and plant scales.
- Understand the changes in microbial community structures through the evaluation of different operational parameters to maximize biogas production.
- Evaluate the potential use of acclimated communities to degrade energy-rich content substrates without pre-treatment stages.
- Explore the influence of the feedstock composition over microbial communities in anaerobic digesters.

The specific objectives of each chapter are summarized below:

Chapter 4

- Develop a workflow to characterize microbial communities using high-throughput sequencing techniques and the downstream analysis using bioinformatics tools.
- Characterize the effect of the co-digestion of food waste and urban sewage over microbial community.

- Identify the microorganisms related to the increase in the biomethanization observed in the anaerobic plant.

Chapter 5

- Characterize the microbial community established in a thermophilic bioreactor to recover energy from raw microalgae.
- Explore the effect of organic loading rate over the microbial community structure.

Chapter 6

- Evaluate from a microbial ecology point of view the use of rumen biomass as a source of hydrolytic microorganisms in anaerobic digesters.
- Identify the microbial community structures established in the rumen bioreactor during raw microalgae anaerobic digestion.
- Analyze the changes in the microbial population structure under operation at different organic loading rates.

Chapter 7

- Identify a microbial core for mesophilic microalgae digestion and co-digestion.
- Understand the differences in the microbial community diversity and structure and their relation with the co-digestion.

Chapter 8

- Elucidate the active microbial community established during thermophilic and mesophilic anaerobic co-digestion of microalgae.
- Explore the influence of the microbial community associated to the sources of feedstock over the bioreactor population diversity and structure.

3. Thesis Plan

3. Thesis plan

The doctoral thesis document is presented in a paper format composed of four published chapters and an additional chapter, also structured as an article. The experimental design of the five chapters has been elaborated to accomplish the aims and objectives of this research work.

Chapter 4

Published in *Journal of Environmental Management*[*].

This chapter investigates the community shifts in an anaerobic membrane pilot plant (AnMBR) treating sewage prior, during and after its co-digestion with food waste. This was the first high-throughput sequencing project developed in the research team. The methodology required for microbial ecology analysis was assembled before this study, including the acquisition of knowledge on bioinformatics. The outcome of Chapter 4 was used to raise the experimental planning of Chapters 5, 6 and 7.

Chapter 5

Published in *Algal Research*[*].

This chapter identifies the composition of a thermophilic hydrolytic acclimated community for raw microalgae degradation. The effect of organic loading rate in a continuous stirred tank reactor (CSTR) configuration operated at a fixed solids retention time (SRT) over microbial structure is here evaluated. The outcome of Chapter 5 was used to support the experimental design of Chapter 8.

Chapter 6

Published in *Bioresource Technology*[*].

This chapter studies the use of a natural hydrolytic consortium such as ruminal fluid to degrade microalgae without pre-treatments and its over-time persistence in an anaerobic membrane bioreactor (AnMBR) operating at different SRT values. Besides, the effect of organic loading rate (OLR) over microbial community diversity is here reported. The outcome of Chapter 6 was used to support the experimental design of Chapter 8.

Chapter 7

Published in *Science of the Total Environment*[*].

This chapter compares the microbial community structures of two systems producing biogas from raw microalgae. The effect of co-digestion and the type of microalgae fed to the anaerobic systems (both AnMBR) is here revealed. A microbial core for microalgae digestion is here elucidated. The outcome of the chapter was used to improve the experimental design of Chapter 8.

Chapter 8

Prepared for future submission.

This chapter identifies the microbial groups of a pilot plant producing biogas from microalgae under thermophilic and mesophilic conditions, discriminating between the present and active members. This chapter also explores the diversity of the influent and its effect over the acclimated microbial community of the bioreactor. The outcome of Chapter 8 allowed to corroborate the microbial core elucidated during the whole research work for energy-rich substrates conversion into biogas.

Chapter 9

This chapter details and synthetizes the overall Ph.D. research outputs and compiles the contributions to the knowledge of this work.

This dissertation finalizes with the Conclusions and Future Perspectives for this research area and includes the Nomenclatures and Abbreviations (Appendix A), the List of Figures (Appendix B) and a List of Tables (Appendix C) to facilitate the comprehension of this work to their readers. Finally, an extended abstract written in Spanish (Appendix D) has been included to meet the regulations of the University of València for theses written in a different language than Spanish or Valencian.

**Journal Editor has permitted the use of the article in this dissertation document*

4. Influence of Food Waste addition over microbial communities in an Anaerobic Membrane Bioreactor plant treating urban wastewater

4. Influence of Food Waste addition over microbial communities in an Anaerobic Membrane Bioreactor plant treating urban wastewater

Abstract

Notorious changes in microbial communities were observed during and after the joint treatment of wastewater with Food Waste (FW) in an Anaerobic Membrane Bioreactor (AnMBR) plant. The microbial population was analysed by high-throughput sequencing of the 16S rRNA gene and dominance of Chloroflexi, Firmicutes, Synergistetes and Proteobacteria phyla was found. The relative abundance of these potential hydrolytic phyla increased as a higher fraction of FW was jointly treated. Moreover, whereas Specific Methanogenic Activity (SMA) rose from 10 to 51 mL CH4 g^{-1} VS, Methanosarcinales order increased from 34.0% over 80.0% of total Archaea, being Methanosaeta the dominant genus. The effect of FW over AnMBR biomass was observed during the whole experience, as methane production rose from 49.2 to 144.5 L $CH_4 \cdot kg^{-1}$ influent COD. Furthermore, biomethanization potential was increased over 82% after the experience. AnMBR technology allows the established microbial community to remain in the bioreactor even after the addition of FW, improving the anaerobic digestion of urban wastewater.

Keywords

anaerobic digestion; AnMBR; biogas; food waste; Illumina; microbial community.

Publication

Zamorano-López, N., Moñino, P., Borrás, L., Aguado, D., Barat, R., Ferrer, J., Seco, A., 2018. Influence of food waste addition over microbial communities in an Anaerobic Membrane Bioreactor plant treating urban wastewater. J. Environ. Manage. 217, 788–796. doi.org/10.1016/j.jenvman.2018.04.018

4.1.Introduction

The anaerobic digestion (AD) of waste has become popular due to its environmental sustainability, as it not only reduces waste production, but also enables bioenergy production (Mao *et al.,* 2015). Methane-rich biogas is produced during the degradation of organic matter through different microbiologically-controlled stages, such as hydrolysis, fermentation, acidogenesis and methanogenesis.

An Anaerobic Membrane Bioreactor (AnMBR) decouples the hydraulic retention time (HRT) from the sludge retention time (SRT), allowing the application of AD to low strength wastewaters treatment, such as urban wastewater (WW). This technology has a suitable effect over AD of WW even when treating urban influents with high concentration of sulfates, which can lead to low methane yields (Giménez *et al.*, 2011). Moreover, the use of membrane technology provides full biomass retention in the digester with reasonable digester volumes, enhancing the heterogeneity of the system and improving domestic WW treatment (Smith *et al.,* 2015).

The AD of food waste (FW) can also contribute to reducing the amount of organic wastes sent to landfills, as required by the European 1999/31/CE Directive. Also, this enhanced version of AD can be a proper way for food disposal and comply with the European 98/2008/CE Directive. Incorporating the FW into the WW influent for joint treatment via AD can improve energy recovery and has other benefits, such as savings in municipal solid waste transportation, reducing fossil fuel consumption and landfill volumes (Kujawa-Roeleveld *et al.,* 2006). The small carbon footprint of food waste disposers and associated water consumption have been reviewed by Mattsson and co-workers (2015). Several studies have addressed the treatment of FW (Fisgativa *et al.,* 2017; Vrieze *et al.,* 2015). However, only a few have focused on AnMBR (Galib *et al.,* 2016) to convert this organic enhanced waste stream into energy.

Microbial population in AD processes provides valuable information and must be considered jointly with process parameters monitoring (Tan *et*

al., 2016). A heterogeneous pool of molecular biological tools can be used to characterize microbial populations. Next generation sequencing (NGS) has especially changed the study of microbial ecology in complex environments such as anaerobic digesters, being Illumina the most applied sequencing technique, due to its reduced cost and the useful information it provides on the microbial population. High-throughput sequencing of biomarkers such as the 16S rRNA gene is a valuable tool for the identification and quantification of key microbial groups in AD (Bartram *et al.,* 2011; Degnan and Ochman, 2012; Vanwonterghem *et al.,* 2014b).

Most previous studies have focused on the methanogenic population of anaerobic digesters, due to its importance in the operational efficiency and energy recovery (Alvarado *et al.,* 2014; Wilkins *et al.,* 2015). However, a global overview of the microbial communities, considering both the *Archaea* and *Bacteria* domains, is needed to understand the implications of these microorganisms in limiting AD steps such as hydrolysis and fermentation. Thus, besides monitoring performance parameters, a thorough analysis of microbial populations with the new molecular tools is needed to better understand AD seeking the improvement of this process management (Carballa *et al.,* 2015).

In this study, a joint treatment of FW and urban WW has been performed in an AnMBR demonstration plant, generating high energy recovery yields in terms of methane and biogas production (Moñino *et al.,* 2017). The notorious improve of the AD of urban WW once the FW addition was over, suggested that microbial population established during the experience was more efficient than the previous one established. Hence, microbial insights of the AnMBR demonstration plant are here explored, revealing the remarkable influence of FW substrate and membrane technology over microbial populations.

4.2.Materials and methods

4.2.1. Demonstration plant

The AnMBR demonstration plant used in this study is situated in the Carraixet WWTP, in Alboraya (València, Spain) (see the process flow diagram in Figure 4.1). The influent for this plant is taken from the pre-treatment of the Carraixet WWTP, after screening and removal of grit and grease. Then, it is treated in a 0.5 mm screen rotofilter, homogenised in the regulation tank (RT) and pumped into a 1.3 m^3 anaerobic reactor (0.4 m^3 head-space volume). This digester is connected to two external membrane tanks of 0.8 m^3 total volume each (0.2 m^3 head-space volume), set in parallel, which allow to do chemical membrane cleaning, or another maintenance operation needed without interrupting the biological process performance. In the membrane tanks, vacuum filtration is applied to obtain the effluent, which is stored in a Clean-in-Place tank. Sludge is continuously recycled from the anaerobic reactor to the membrane tanks and the SRT is controlled by purging a fraction of the sludge from the anaerobic reactor intermittently during the day. A commercial food waste disposer and a 0.5 mm space screen rotofilter are used for the pre-treatment of the FW, which is stored in a co-substrate tank (CT) with a usable volume of 0.180 $m^3$and is also connected to the anaerobic reactor. A three-way valve alternates wastewater and FW inputs from the RT or CT, respectively.

The FW fraction is supplied according to the Penetration Factor (PF) established, which is defined as the percentage of households using food waste disposers. Two scenarios were evaluated, assuming that 40% or 80% of the population were grinding the food FW. These scenarios were explored as they might be feasible in small areas where household food waste disposers can be implemented. According to the national plan for waste management (PNIR 2008-2015), a mean value of 0.63 kg $FW \cdot hab^{-1} \cdot d^{-1}$ is generated in Spain. The Statistical National Institute of Spain reported in 2010 an urban wastewater generation of 282.4 $L \cdot hab^{-1} \cdot d^{-1}$ in 2010 (last available data). From this volume 225.92 $L \cdot hab^{-1} \cdot d^{-1}$ (an 80% approximately) is considered to have a domestic origin. Experimental results

determined that a FW and WW mixture of 2.52 $L \cdot hab^{-1} \cdot d^{-1}$ is generated during FW grinding in household disposers. Hence, a resulting ratio of 11.2 mL of grinded FW per L of WW was fed to the pilot plant: 4.48 and 8.96 mL of FW per L of WW, representing a 40% and 80% PF scenario, respectively.

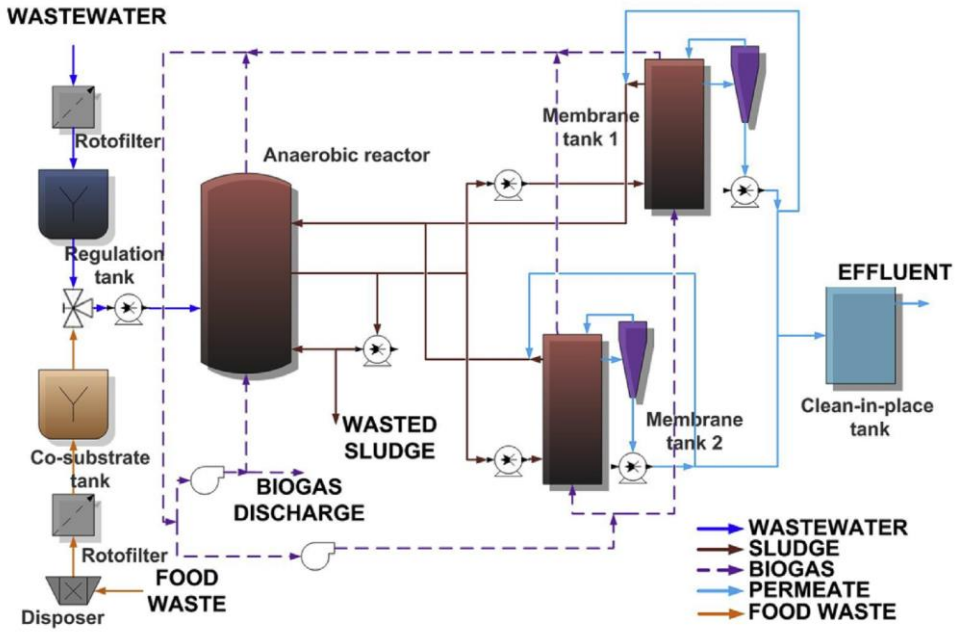

Figure 4.1. AnMBR demonstration plant process diagram.

4.2.2. Operational conditions

Four different pseudo steady-state periods (Table 4.1), determined after stabilising solids concentration and methane production in the AnMBR, were selected for microbial community analysis. In Periods 2 and 3, the AnMBR treated both FW and wastewater substrates at different PF (40 and 80%, respectively). In the remaining periods, only wastewater was treated. Period 1 was prior to the joint treatment and Period 4 was after FW addition, when a new pseudo steady state had been reached.

Table 4.1. Operational conditions of each pseudo steady-state period studied in the AnMBR plant.

	Period 1	Period 2	Period 3	Period 4
SRT (d)	42±2	70±11	69±6	70±2
T (ºC)	25±2	28±1	27±1	28±3
HRT (h)	30±4	22±6	24±6	22±4
PF (%)*	0	40	80	0
Treatment flow ($L \cdot d^{-1}$)	1630 ± 154	2223 ± 516	2038 ± 549	2223 ± 359

*PF was defined as the percentage of households that use food waste disposers.

4.2.3. Biological process monitoring

Influent, effluent and AnMBR reactor samples were collected twice a week to monitor the biological process. Volatile Solids (VS), COD, sulphide and sulfate concentrations were determined according to Standard Methods (APHA and APHA/AWWA/WEF, 2012). Methane production was recorded and dissolved methane in the effluent was calculated by Henry's Law, as described in Giménez *et al.* (2012). Specific methanogenic activity (SMA) tests were carried out for each period using the Automatic Methane Potential Test System (AMPTS) [Bioprocess Control, Sweden] and performed as described in Ozgun *et al.* (2015).

4.2.4. Sample collection and DNA extraction

Sludge samples were collected from the AnMBR at each period (see Table 4.1) and were immediately stored in 1 mL cryotubes at -20ºC to characterize the microbial population involved in the AD process. Extraction of DNA was performed in an E.Z.N.A Soil DNA Kit (Omega-Biotek), according to the manufacturer's protocol but with minor modifications to improve the DNA yield (data not shown): (i) incubation time was increased from 10 to 20 minutes at 70ºC and (ii) the second incubation was at 95ºC for 5 minutes during the cell lysis stage. Extractions were performed from 1 mL of homogenized sludge. A Nanodrop 2000 spectrophotometer (Thermo Scientific) was used to determine the concentration and purity of DNA through the absorbance measured at wavelengths of 260, 230 and 280 nm. In order to avoid contamination by RNA, humic acids or other compounds, only sequences with an $A_{260/230}$ ratio between 2.0 and 2.2 and an $A_{260/280}$ ratio over 1.8 were sequenced.

4.2.5. Illumina amplicon sequencing

A set of libraries from the v4 hyper-variable region of the 16S rRNA gene were prepared according to the procedure described in Caporaso *et al.* (2011). Universal prokaryotic indexed primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'), were used for this purpose according to the following amplification conditions: denaturing stage at 95ºC during 30 seconds, 28 cycles of 30 seconds steps at 95, 55 and 72ºC, successively; and final elongation stage at 72ºC during 5 minutes. The concentration of DNA in selected samples was determined in a Qubit 3.0 fluorometer (Life Technologies) and 0.2 ng/μL of each DNA sample were used for library preparation with indexed primers. The resulting amplicons were multiplexed in a Nextera XT Index Kit (Illumina) and sequencing was performed according to the Illumina manufacturer's protocol in a MiSeq reagent kit V3, on a MiSeq sequencer in a 2x300 bp paired-end run, in genomic department of the Fundación para el Fomento de la Investigación Sanitaria y Biomédica de la Comunidad Valenciana (FISABIO).

4.2.6. Illumina processing data and statistics

Raw data retrieved from Illumina sequencing after barcode and index removal was sequentially processed through the following pipeline: first, the prinseq-pl algorithm (Schmieder and Edwards, 2011) was applied at the trimming stage, within a quality-threshold of 30 and a window length of 12 bp. The trimmed paired-end reads were merged together with default parameters of fastq-join (Aronesty, 2011), and checked for chimeras within the UCHIME algorithm (Edgar *et al.*, 2011). Non-chimeric sequences were classified up to genus level, applying a confidence threshold of 0.8, in the Ribosomal Database Project's Classifier tool release 2.11 (Cole *et al.*, 2009). R-software and the Vegan v.2.3-1 package (Oksanen *et al.*, 2016) were used to estimate relative abundances between samples and to calculate diversity and evenness indexes: Shannon-Wiener and Simpson (expressed as inverse Simpson for a better comparison with the Shannon-Wiener index). Raw

sequences have deposited as follows: Database: BioProject (PRJNA339420, samples SRS2046188, SRS2046189, SRS2046190 and SRS2046191).

4.3. Results and discussion

4.3.1. AnMBR demonstration plant performance.

The FW treated in this study was mainly characterized by high carbohydrate content and a remarkable presence of polysaccharides, according to the following frequency of occurrence: rice (88%), fruit remains and peel (80%), potatoes (68%), bread (64%), pasta (56%), seafood (52%), cooked vegetables (44%), chicken (32%), salad (16%), fish (16%), pork (8%) and beef (8%). The exhaustive characterization performed in Moñino *et al.* (2016), showed that ground FW presents high COD (100 times higher than the average concentration in WW) and small size (90% of the particles under 0.5 mm), allowing a significant part of the FW to reach the AnMBR despite the restrictive pre-treatment of 0.5 mm sieve. This substrate can be more easily hydrolyzed than WW, as demonstrated by previous assays which shown 72% of anaerobic biodegradability, leading to increase the production of volatile fatty acids (VFAs) and other fermentation by-products in the digester. The higher production of compounds like acetate or hydrogen enhances the system's methane production potential, as they are substrates available for MA, whose can finally reduce them to methane.

Four pseudo steady-state periods were defined according to the stabilized concentration of VS in the AnMBR (see Table 4.2). The COD and sulfate concentrations for each period are also shown in Table 4.2 for WW, FW and the total concentration of the influent. It is remarkable that, due to the FW addition, the COD concentration in the influent increases while the sulfate concentration remains in the same range, in Periods 2 and 3. Consequently, the COD/S-SO_4 ratio was increased in the co-treatment periods. Effluent concentrations in the plant were similar in all periods and lower than the limit concentration allowed to accomplish the discharge requirements (125 mg COD $\cdot L^{-1}$), according to Council Directive 91/271/EEC of 21 May 1991. The excellent retention capacity of the

membranes made the system capable of achieving high effluent quality in all periods. Regarding membrane fouling, no meaningful differences were observed between the different operating conditions when feeding WW in comparison with treating WW jointly with FW.

Table 4.2. Performance and biological process monitoring of AnMBR plant.

	Period 1	Period 2	Period 3	Period 4
Influent characteristics				
FW COD (mg COD·L^{-1})	-	69455 ± 20130	71872 ± 16518	-
WW COD (mg COD·L^{-1})	560 ± 64	618 ± 185	564 ± 182	541 ± 188
Influent COD (mg COD·L^{-1})		797 ± 205	989 ± 206	
FW S-SO_4 (mg S·L^{-1})	-	224 ± 96	140 ± 55	-
WW S-SO_4 (mg S·L^{-1})	98 ± 26	114 ± 13	123 ± 16	124 ± 19
Total influent S-SO_4 (mg S·L^{-1})	-	114 ± 15	123 ± 18	-
Ratio COD/S-SO_4 (mg COD·L^{-1}/ mg S-SO_4·L^{-1})	5.1 ± 0.8	7.0 ± 1.9	8.0 ± 1.9	5.6 ± 1.7
VS reactor (mg·L^{-1})	11444 ± 650	10873 ± 340	9997 ± 419	8907 ± 271
Total CH_4 (L·kg^{-1} influent COD)	49.2 ± 16.9	110.7 ± 53.5	144.5 ± 45.8	89.3 ± 39.7
Increase in methane production over Period 1 (%)	-	125%	193%	82%
SMA (mL CH_4 ·g^{-1} VS)	10.44 ± 0.12	49.22 ± 0.54	50.98 ± 0.86	43.59 ± 0.36
COD removal by MA*	111.6 ± 33.2	266.9 ± 70.4	345.6 ± 65.5	210.3 ± 32.2
COD removal by sulfate-reducers**	320.6 ± 45.7	274.0 ± 52.1	214.7 ± 29.8	290.0 ± 65.8

* Calculated as g COD transformed into methane · kg^{-1} influent COD

** Calculated as g COD transformed by sulfate-reducers · kg^{-1} influent COD

4.3.2. Methanogenic potential in the AnMBR demonstration plant.

The COD removed during AnMBR operation, besides the COD purged out of the system, can be attributed to two different biological controlled pathways, i.e. (i) sulfate reduction and (ii) methanogenesis. For the COD removed by sulfate-reducers calculation, it was assumed that 2 g COD·g^{-1} S reduced are consumed by sulfate-reducers. Contribution of sulfate-reducers to COD removal ranged between 290.0 and 320.6 g COD · kg^{-1} influent COD when only WW was treated and 214.7 and 274.0 g COD·kg^{-1} influent COD in co-treatment periods.

Methane contained in the biogas and dissolved in the effluent was measured daily and used for calculation of the COD removal. The average results for each period are shown in Table 4.2. Lower yields of COD removal were found in Period 1 due to the characteristically low organic load of urban wastewater. Then, organic load was increased by the addition of FW to the plant influent, while the concentration of sulfates remained stable during the

whole period (further details can be found in Moñino *et al.*, 2017). Hence, the observed change in Period 2 and 3 of COD/S-SO_4 ratio (expressed in terms of mg COD/mg S-SO_4) is mainly attributed to the addition of this rich source of organic matter (see Table 4.2). Furthermore, as it has recently been indicated by Paulo *et al.* (2015), acetoclastic methanogenesis co-exists with the sulfate-reduction of intermediate AD products when influent COD/S-SO_4 ratios are over 3-4 mg COD/mg S-SO_4. The COD/S-SO_4 ratio in the AnMBR influent increased to 7 and 8 mg COD/mg S-SO_4 due to the FW addition in Periods 2 and 3, respectively. This phenomenon boosted methanogenic pathways in the AnMBR during joint FW and WW treatment: 266.9 g COD·kg^{-1} influent COD in Period 2 and 345.6 g COD·kg^{-1} influent COD in Period 3 were transformed into methane. These results evidence the favorable effect of FW on the whole AD process.

The longer the SRT the higher the methane production in AnMBR. The substrate is retained in the system for longer, allowing higher levels of hydrolysis and the consequent increased degradation of slowly biodegradable organic compounds. Under these operational conditions there is a longer contact time between the particulate fraction of the organic matter and the enzymes responsible for its hydrolysis, leading to a higher concentration of hydrolyzed products that can be converted into VFAs, which are suitable substrates for MA. Related to this fact, an increase of the measured Specific Methanogenic Activity from 10 to 51 mL CH_4·g^{-1} VS·day^{-1} was observed at 42 days and 70 days SRT, respectively (see Table 4.2). Methane production for each period is also shown in Table 4.2, which rose to 125 and 193% in Periods 2 and 3, over Period 1. Nevertheless, the joint treatment of different substrates leads to a synergetic effect, so that, besides the longer SRT, these results suggested the proliferation of a different microbial population while the FW was being added to the AnMBR plant influent. Further experimental support via high-throughput sequencing of 16S rRNA amplicons was therefore required to better understand the influence of FW substrate on AnMBR microbial populations.

4.3.3. 16S rRNA gene amplicon sequencing

Illumina sequencing of 16S rRNA amplicons performed in this study allowed a thorough analysis of the microbial community established in the AnMBR. Sludge samples were collected in the four pseudo-steady state periods when VS concentration was stabilized. A total amount of high-quality Illumina reads ranging from 19,321 to 33,556 sequences per sample and 293±24 bp mean length were obtained and the taxonomy was assigned within the RDP Classifier tool. After application of a 0.8 confidence-threshold a total amount of 825, 652, 761 and 711 genera in Periods 1,2,3 and 4; was respectively found. The percentage of the *Archaea* or *Bacteria* genera identified is shown in Table 4.3. Most of the sequences retrieved were assigned to the *Bacteria* domain and exceeded 96% in all the periods, while the remaining sequences belonged to the *Archaea* domain, reaching a maximum of 3.4%.

Table 4.3. Relative abundance at domain taxonomic level of the sequences retrieved, sulfate-reducers, methanogenic Archaea (MA) and diversity and evenness indexes in each pseudo steady-state period of the AnMBR.

	Period 1	**Period 2**	**Period 3**	**Period 4**
Archaea (%)	0.9	2.3	3.4	3.2
Bacteria (%)	99.1	97.7	96.6	96.8
Sulfate-reducers (%)	4.6	1.5	2.8	3.1
MA (%)	0.5	2.2	3.0	2.9
MA/sulfate-reducers ratio	0.1	1.4	1.1	1.0
Shannon Index	3.6	3.0	3.1	3.5
Inverse Simpson Index	13.7	5.8	5.9	9.2

4.3.4. *Characterisation of microbial population in the AnMBR*

A complex and heterogeneous microbial population was characterized in the AnMBR during the joint FW and WW treatment experience. The different *Archaea* orders and *Bacteria* phyla detected in the study are shown in Figures 4.2a and 4.2b with their relative abundances. As it can be seen in this figure, a shift in the microbial population in the AnMBR appeared as the FW was being added to the influent (Period 2). The main difference with

respect to Period 1 was found in the composition of the methanogenic population, as it changed from a heterogeneous combination of acetoclastic, hydrogenotrophic and or methylotrophic methanogens to a community dominated by acetoclastic-capable *Archaea* orders like *Methanosarcinales*.

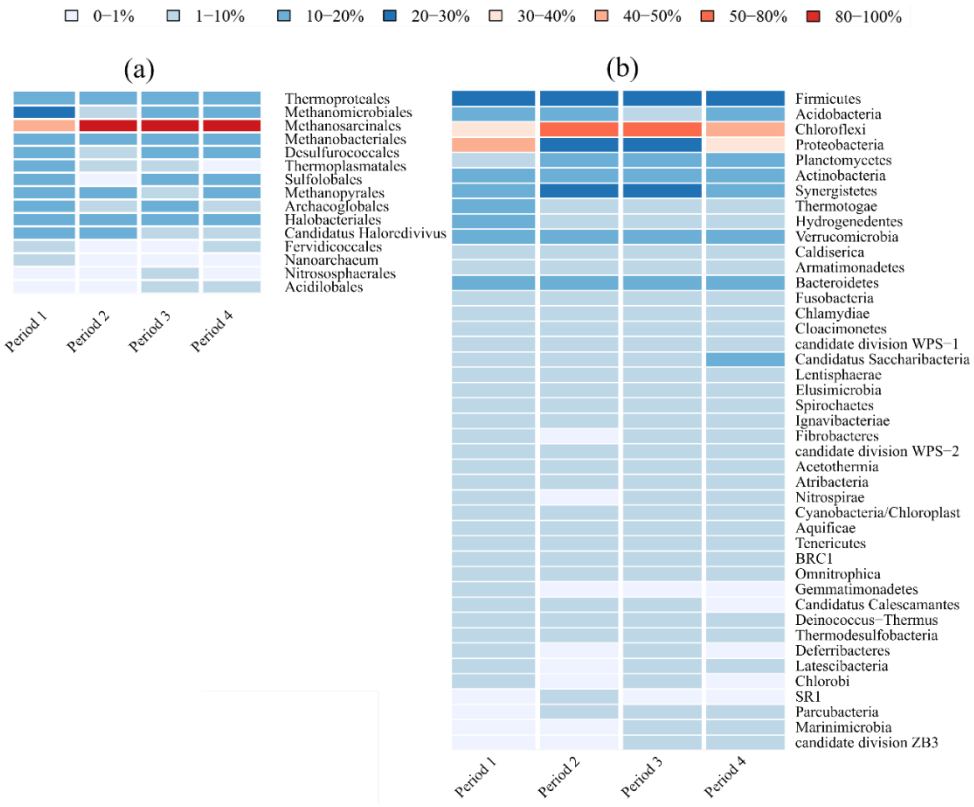

Figure 4.2. Heatmap showing the composition of microbial community at (a) *Archaea* order and (b) *Bacteria* phyla taxonomic levels.

a. Methanogenic Archaea

Methane production in AD relies on the activity of the methanogenic *Archaea* (MA) population. These microorganisms grow on a narrow spectra of substrates like H_2 and CO_2 or formate (hydrogenotrophic methanogenic *Archaea*, H_2MA), although some MA can also reduce a wider spectra of substrates to methane, including here acetate (acetoclastic methanogenic

Archaea, AcMA) or methylated compounds (methylotrophic methanogenic *Archaea*, MeMA) (Lyu and Lu, 2015). Most of the methanogenic orders detected in the AnMBR were H_2MA (*Methanobacteriales*, *Methanopyrales*, *Methanomicrobiales* and some *Methanosarcinales)*, although some AcMA belonging to the *Methanosarcinales* order were observed (Table 4.4, Supplementary Information). In Period 1, a heterogeneous community, slightly dominated by *Methanosarcinales* (34.0% of total MA) and *Methanomicrobiales* (13.6%) was detected. In the joint FW and WW treatment period a change in MA population took place. The *Methanosarcinales* order was clearly favored, from 34.0% in Period 1 to 87.1% in Period 2. The relative abundance of this order, in which H_2MA and AcMA have been reported, remained at values over 82.0% in the subsequent pseudo steady-state periods analyzed.

The *Methanosarcinales* order contains AcMA genera such as *Methanosaeta*, whose relative abundance, considering the total amount of genera (including *Bacteria* and *Archaea*) increased from 0.2 in Period 1 to 1.9%, 2.7% and 2.5% in Periods 2, 3 and 4, respectively. In Figures 4.3a and 3b, the relative abundance of *Archaea* and *Methanosaeta* is shown, describing a proportional increase. It therefore seems that the increase in *Archaea* is due to the notable relative increase in the *Methanosaeta* genus from 57.0% to percentages higher than 82% (Table 4.4). The enrichment of this genus in the AnMBR may also explain the increased SMA values recorded in Periods 2 and 3 during joint FW and WW treatment (see Table 4.2). Di Maria and Barrata (2015) also detected high relative abundance of this genus when co-digesting sludge with food wastes (mainly composed of potato, fruit and vegetables). *Methanosaeta* was also dominant in a recently reported study on anaerobic waste food digestion in a mesophilic reactor (Zamanzadeh *et al.*, 2016).

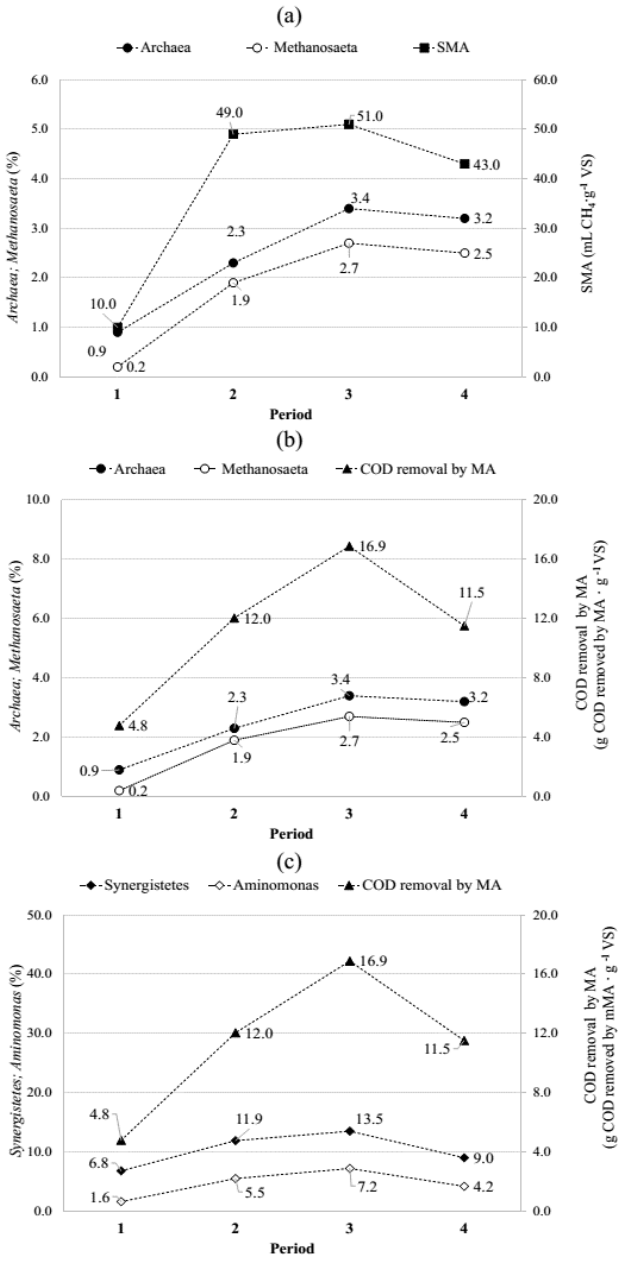

Figure 4.3. Evolution of: (a) relative abundance of *Archaea* and *Methanosaeta* related to SMA values or (b) COD removal by MA, and (c) relative abundance of *Synergistetes* phylum and related-genus *Aminomonas* with respect to COD removal by MA. Relative abundances are referred to the total amount of sequences retrieved.

Relative abundances of relevant genera with respect to the total amount of sequences (including both *Archaea* and *Bacteria* domains) were calculated for trend comparison to relevant operational parameters such as SMA and COD removal and shown in Figures 4.3a, 4.3b and 4.3c. Comparing the *Archaea* and *Methanosaeta* percentages of relative abundance with the SMA (Figure 4.3a), the same trend can be observed between the relative abundance of *Methanosaeta* and SMA values, suggesting the implication of this genus in the increased biomethanization capacity of the system. In Figure 4.3b, the same percentages are plotted against COD removal by MA. According to this figure, proportional increases of both COD removal and *Methanosaeta* relative abundance can be observed from Period 1 to Period 2 and subsequently to Period 3. However, these trends differ in Period 4, when COD removal starts to fall. In this case, the biodegradability of the influent decreased once FW addition had finished and interfered in the AD hydrolysis and fermentation stages. The conditions established in the AnMBR system seem to be favorable to the last AD step, as the considerable increase in methanogenic activity with 44 mL $CH_4 \cdot g^{-1}$ VS is maintained in Period 4. The advantages of membrane technology should be considered here, as the total retention of the biomass achieved in AnMBR systems also allows suspended microorganisms to remain in the digester, unlike the gravity separation systems. In this study, the addition of FW substrate first enhanced the population's key methanogenic microorganisms, such as those belonging to *Methanosarcinales* order. Yet, their presence in the system remained stable in the last pseudo steady-state period, after the FW addition experience and more than 70 days of operation, explaining the remarkable methanogenic activity obtained. Further studies on important functional genes in methanogenesis, such as the mcrA gene (Alvarado *et al.*, 2014), may be able to link the relative abundance and role of *Methanosaeta* and other MA to the higher values of SMA observed (see Table 4.2).

a. Bacteria

Community analysis of AnMBR sludge samples revealed a major composition of *Bacteria* belonging to four main phyla: *Chloroflexi*, *Firmicutes*, *Synergistetes* and *Proteobacteria*. This bacterial group can explain itself almost 80% of the microbial diversity detected in each period. Table 4.4 details the genera composition of each relevant phylum, according to their relative abundance and considering the total amount of genera identified for each phylum in the AnMBR. Only phyla that represented at least 10% of the bacterial community in any period are given in this table and have been thoroughly explored.

Chloroflexi microorganisms have been widely detected in mesophilic anaerobic digesters (Di Maria and Barratta, 2015; Sundberg *et al.*, 2013; Yi *et al.*, 2014). In the present study, these bacteria seem to play an important role in the anaerobic digestion of FW, as their presence was the highest detected in Periods 2 and 3, when 40% and 80% PF of FW was digested together with WW: 41.2% and 41.3% of relative abundance of the *Bacteria* domain, respectively. The most abundant *Chloroflexi* genus was *Levilinea*, a mesophilic microorganism that has been reported as a common fermenter from a variety of substrates like sugars and peptides (Yamada *et al.*, 2006). The relative abundance of *Levilinea* dropped slightly in Periods 2, 3 and 4 to approximately 64% of the relative abundance in Period 1 (70.4%). Competition against other *Chloroflexi* phylum members (*Bellilinea* or *Longilinea*) may explain this minor difference. This enhancement of bacteria belonging to the *Chloroflexi* phylum is in agreement with the previously reported study by Di Maria and Barratta (2015), in which *Longilinea* was also detected during the mesophilic anaerobic co-digestion of sludge with fruit and vegetable waste. In this study, a high percentage of occurrence of cellulose-containing wastes was also found. A recent study reveals the ability of *Anaerolineae* class microorganisms, such as *Bellilinea* and other bacteria classified in the *Chloroflexi* phylum, to attach to cellulose, which leads to a competitive advantage in complex environments (Xia *et al.*, 2016). According to the present study, the dominance of *Chloroflexi*

microorganisms capable of cellulose degradation may have boosted COD removal during joint FW and WW treatment.

Diverse genera belonging to the *Firmicutes* phylum were also detected in the AnMBR sludge samples. Genus *Coprothermobacter* suffered a remarkable fourfold decrease after the addition of FW in Period 2. This genus is reported to participate in fermentation pathways in which hydrogen is released (Sun *et al.,* 2015) and may be syntrophically linked to H_2MA. *Clostridium* is another important genus that belongs to *Firmicutes* and is commonly found in anaerobic digesters. Species belonging to this genus widely produce acetate as a main sub-product during their fermentation pathways (Yutin and Galperin, 2013). Once again, a link between the fermentation stage by acidogenic bacteria and the acetoclastic pathway that characterizes the *Methanosaeta* genus seems to be related. Moreover, this relationship also links to the higher SMA observed in Periods 2 and 3.

Less abundant genera belonging to the *Firmicutes* phylum, like *Garciella* and *Lactobacillus*, should also be considered, due to their involvement in the fermentation stage of carbohydrates and more complex substrates. Indeed, *Lactobacillus* has been reported to degrade cellulose-related products (Sträuber *et al.,* 2016). However, its relative abundance in the AnMBR was only noticeable in Period 4, suggesting that this genus was surpassed by other microorganisms with a higher affinity to the FW composition, like the *Chloroflexi* phylum. However, when only treating WW a slight fluctuation in microbial population was detected, *Lactobacillus* was the dominant genus in the *Firmicutes* phylum.

The phylum *Synergistetes* was also significantly abundant in the AnMBR throughout the experimental period, exhibiting values close to 10% of the total *Bacteria* detected. The amino acid degradation capacity of microorganisms belonging to the *Synergistetes* phylum has been previously reported by Hugenholtz *et al.* (2009). Related to this phylum, *Aminiphilus*, *Aminobacterium* and *Aminomonas* genera were detected in the AnMBR. The *Aminomonas* microorganisms thrived during the joint FW and WW

treatment and were the most abundant *Synergistetes* genus, whose relative abundance rose to 55.4% in Period 3, when the maximum amount of FW was being treated in the plant. The higher biodegradability of the influent in Periods 2 and 3 (due to the FW), might promote the hydrolysis of proteins with the consequent release of amino acids, which were finally fermented into small carbon compounds and VFAs by *Synergistetes* belonging microorganisms. This phenomenon seems to explain the similar trend shown in Figure 4.3c, which plots COD removal by MA against the relative abundance of this phylum and the dominant *Aminomonas* genus. The release of these fermentation by-products seems to also favor the methanogen population, thus supporting the noticeable enhancement of SMA values and COD removal in terms of methane production in Periods 2 and 3.

The *Proteobacteria* relative abundance fell during joint FW and WW treatment. As this is a widely diverse taxonomic group, the microorganisms belonging to it are involved in different metabolic reactions. Their suitability for the degradation of polysaccharides has recently been reported when operating an AnMBR treating cellulose-enriched sewage (Watanabe *et al.*, 2016). The simultaneous presence of *Proteobacteria* and *Chloroflexi* phyla may therefore play an important role in FW degradation. Proteobacteria microorganisms are not only involved in AD hydrolysis and fermentation steps, but also in the degradation of intermediate products using sulfate, sulfite or thiosulfate as electron acceptors (El Fantroussi *et al.*, 1997), which are present in the AnMBR plant influent.

4.3.5. Co-existence of sulfate-reducing microorganisms with methanogens in the AnMBR

Even when high concentrations of sulfate in the influent are found, acetoclastic methanogenesis can prevail despite of the suitable pathway of VFAs degradation by sulfate reducers. As mentioned in Section 4.3.2, the COD/S-SO_4 ratio was enhanced during the joint treatment periods due to the FW addition. This change boosted methanogenic metabolic pathways as it was also observed in terms of COD removal (Table 4.2). Different known sulfate-reducers, such as *Desulfurococcales*, *Sulfolobales* (*Archaea*

domain), *Desulfarculales, Desulfobacterales, Desulfovibrionales, Desulfobacteriales, Desulfuromonadales, Thermodesulfobacterales and Desulfaculales* (*Bacteria* domain), were detected in the AnMBR. The relative abundance percentage of these sulfate-reducer orders was compared to MA orders, like *Methanobacteriales, Methanomicrobiales, Methanopyrales* and *Methanosarcinales* (see Table 4.3). The *Desulfomonile* relative abundance rose from 1.0% in Period 1 to 14.2% in Period 2 and remained at similar relative abundance values inside this phylum, being the dominant sulfate reducer genus identified in the AnMBR. The MA/sulfate-reducers ratio was calculated and revealed a considerable rise in Periods 2 and 3, coinciding with FW addition. Hence, in the AnMBR plant the co-existence of AcMAs like *Methanosaeta* with sulfate-reducers was observed, not only contributing to high removal of COD but also to high biomethanization values.

4.3.6. *Diversity analysis of the AnMBR population*

The richness and evenness of the AnMBR system was estimated by the Shannon-Wienner and Simpson indexes (Table 4.3). The loss of diversity in the reactor was observed during the joint treatment of FW, according to the similar decreasing trends of both estimations. The common range of the Shannon-Wienner index is between 1.5 and 3.5 and therefore the high values obtained in this study between 3.0 (Period 2) and 3.6 (Period 1), support the huge complexity of the AnMBR. As it is observed in this study, increased SRT provided by membrane technology operation allows the system to retain a widely diverse biomass, increasing the heterogeneity of the system. Estimating the diversity indexes gives a better understanding of the specialization of the biomass.

Lower values of Shannon-Wiener and inverse Simpson diversity indexes were obtained as a higher fraction of FW was treated in the AnMBR. Evenness of some of the *Chloroflexi* and *Synergistetes* phyla also increased during Period 2 and 3. Although advantageous effects of increased SRT might contribute to AnMBR population changes observed, the additional organic matter source, *i.e* FW; acted as a selection factor over the AnMBR

biomass by shaping the microbial community. This trend is strongly supported by the estimation of the inverse Simpson dominance index, whose value decreased as a higher fraction of FW was added to the influent. Finally, when FW was no longer supplied (Period 4), both diversity indexes remained lower than those obtained in Period 1. These results support the long-term establishment of a FW-degrading community in the reactors. Membrane technology allowed the efficient microbial community established during FW treatment to remain in the AnMBR, improving its capacity for AD from urban WW.

4.3.7. *FW-degrading microbial population in the AnMBR*

In this study the addition of FW to the influent of the AnMBR plant enhanced the SMA of the system and the methane content of the biogas. Accordingly, the substrate composition shifted the AnMBR microbial population in Period 1 to the community detected in Periods 2 and 3, when a different amount of FW was treated. The FW-degrading microbial population was thus established during the joint treatment experience in the AnMBR plant and subsequently remained. Substrate composition and SRT have been reported to strictly control AD microbial communities (Vanwonterghem *et al.*, 2015).

The FW-degrading community detected here has a strong hydrolytic and fermentation potential, which seems to be related to the dominance of the *Chloroflexi* phylum. The presence of genera like *Longilinea*, *Levilinea*, *Lactobacillus* or *Garciella*, and the higher composition of carbohydrates and complex polysaccharides of FW seem to be closely related (Yi *et al.*, 2014). The fermentation of the hydrolyzed protein content in the AnMBR influent during FW treatment was remarkable, due to the spread of more peptide fermenters like those belonging to the *Synergistetes* phylum such as *Aminomonas*. An efficient set of fermentation reactions, driven by the microbial community established in the AnMBR, would have led to higher production of key intermediate AD products, such as hydrogen or acetate in the system. The efficient transfer of these by-products, especially when they are reduced to acetate, allowed the dominance of acetoclastic *Methanosaeta*

members inside the MA population, enhancing methanogenic activity in the AnMBR.

Interestingly, the FW-degrading population remained in the AnMBR when this substrate was no longer being added to the plant influent, *i.e.* in Period 4. Although minor changes in the relative abundance of important FW-degrading phyla were found in Period 4, *Chloroflexi* and *Synergistetes* remained at high relative abundance values in the AnMBR. The composition of the microbial population detected in the AnMBR 70 days after stopping the joint treatment (Period 4), strongly supports the reported improvement in the anaerobic treatment of WW in this plant. According to the high SMA obtained (43 vs 10 mL $CH_4 \cdot g^{-1}$ VS) and the 82% increase in methane production between Periods 1 and 4 (see Table 4.2), the resulting microbial population found in the AnMBR shows a remarkable potential for high energy recovery from WW. However, further research is needed for a better understanding of their metabolic implications for the AD process. Metaproteomics jointly with metagenomics approaches could reveal important information of the degrading potential of this community, dominated by *Chloroflexi*, *Firmicutes*, *Proteobacteria* and *Synergistetes*. This would also reveal how the AD processes can be improved through the addition of highly biodegradable substrates such as FW during a short period. With this strategy, limiting steps such as hydrolysis are overpassed and, consequently, suitable effects among the whole AD steps finally lead to an efficient conversion of organic matter and a remarkable recovery of energy.

In this study the characterization of microbial composition in an AnMBR was reported after Illumina sequencing of v4 hyper-variable region 16S rRNA amplicons. According to the results obtained, the substrate has a strong influence on microbial population dynamics in anaerobic digesters. Although membrane technology can enhance the diversity of sludge microbial communities, the substrate acts as a selective factor, resulting in specific substrate-degrading communities. This substrate-dependent population is characterized by lower diversity but has a remarkable effect on

urban WW treatments. These significant results should be considered in the future management of anaerobic urban wastes, as they reveal the possibility of boosting microbial populations with rich and easily degradable organic substrates.

The combination of a substrate with a high organic matter content like FW in a digester configuration such as an AnMBR, in which biomass is highly concentrated, produced a selection of microorganisms with a wide ability to efficiently degrade organic matter from different sources. Furthermore, the stability provided by the AnMBR configuration in long-term operations in this pilot-plant allowed the microbial population that had been established to remain in the digester, thus improving the performance in treating urban WW. These results show that monitoring microbial responses to operational conditions is not only necessary for a better understanding of the AD process but is also essential to improve its management.

Conclusions

A remarkable change in the original microbial population was detected through Illumina 16S amplicon sequencing of an AnMBR demonstration plant during joint FW and WW treatment. Phyla with high hydrolytic and fermentation potentials (41.2%-41.3% *Chloroflexi*, 10.8%-10.8% *Firmicutes*, 17.4%-18.3% *Proteobacteria* and 11.9%-13.5% *Synergystetes)* and acetoclastic methanogens like *Methanosaeta* thrived during joint treatment. The establishment of this population as the FW fraction increased, enhanced the SMA from 10 to 43 mL CH_4 g^{-1}VS and provided a boost of 82% in methane production. The FW-degrading population was not only established during the joint treatment phase but also remained in the AnMBR, favored by membrane technology, leading to the observed improvement of the anaerobic treatment of urban WW.

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Table 4.4. AnMBR dominant microbial population. Composition of each *Archaea* order or *Bacteria* phyla (bold written) is shown through the relative abundances of each genera inside each mentioned taxonomic level. Only genera with relative abundances over 10.0% in at least one of the four periods have been explored.

	Period 1	**Period 2**	**Period 3**	**Period 4**
Archaea				
Methanosarcinales	**34.0**	**87.1**	**84.4**	**82.1**
Methanosaeta	57.0	95.1	94.5	93.8
Methanosalsum	15.0	0.5	0.9	1.8
Methanomethylovorans	20.0	3.9	3.8	3.9
Methanimicrococcus	5.0	0.3	0.5	-
Methanohalobium	1.0	0.3	-	-
Methermicoccus	2.0	-	0.3	0.6
Methanomicrobiales	**13.6**	**0.9**	**1.1**	**1.4**
Methanospirillum	62.5	25.0	20.0	11.1
Methanosphaerula	5.0	25.0	10.0	33.3
Methanolinea	15.0	-	40.0	22.2
Methanoculleus	10.0	-	-	-
Methanolacinia	2.5	25.0	-	-
Methanomicrobium	5.0	25.0	30.0	11.1
Methanoregula	-	-	-	22.2
Methanobacteriales	**5.8**	**5.4**	**2.1**	**5.6**
Methanosphaera	41.2	12.5	15.0	20.0
Methanobrevibacter	41.2	37.5	40.0	34.3
Methanothermobacter	5.9	-	-	-
Methanobacterium	11.8	50.0	45.0	45.7
Methanopyrales	**5.1**	**1.1**	**0.9**	**1.8**
Methanopyrus	100.0	100.0	100.0	100.0
Bacteria				
Chloroflexi	**23.2**	**41.2**	**41.3**	**32.9**
Bellilinea	12.1	15.3	15.1	14.3
Levilinea	70.4	64.0	64.4	63.9
Longilinea	9.3	11.6	10.4	10.6
Firmicutes	**12.8**	**10.8**	**10.8**	**14.9**
Coprothermobacter	52.2	12.6	9.2	11.5
Clostridium XI	6.3	0.4	14.2	0.3
Lactobacillus	0.1	0.2	0.8	19.5
Garciella	0.1	0.1	0.6	0.7
Proteobacteria	**32.2**	**17.4**	**18.3**	**22.7**
Arcobacter	19.5	0.1	0.6	0.7
Desulfomonile	1.0	14.2	7.8	12.6
Synergistetes	**6.8**	**11.9**	**13.5**	**9.0**
Aminiphilus	10.4	3.4	3.1	7.5
Cloacibacillus	14.4	12.0	9.1	10.2
Aminobacterium	22.6	19.4	20.7	19.4
Synergistes	23.0	16.8	11.1	12.7
Aminomonas	23.4	47.6	55.4	48.2

5. Thermophilic anaerobic conversion of raw microalgae: microbial community diversity in high solids retention systems

5. Thermophilic anaerobic conversion of raw microalgae: microbial community diversity in high solids retention systems

Abstract

The potential of microbial communities for efficient anaerobic conversion of raw microalgae was evaluated in this work. A long-term operated thermophilic digester was fed with three different Organic Loading Rates (OLR) (0.2, 0.3 and 0.4 g·L-1·d-1) reaching 32-41% biodegradability values. The microbial community analysis revealed a remarkable presence of microorganisms that exhibit high hydrolytic capabilities such as *Thermotogae* (~44.5%), *Firmicutes* (~17.6%) and *Dictyoglomi*, *Aminicenantes*, *Atribacteria* and *Planctomycetes* (below ~5.5%) phyla. The suggested metabolic role of these phyla highlights the importance of protein hydrolysis and fermentation when only degrading microalgae. The ecological analysis of the reactor suggests the implication of the novel group EM3 in fermentation and beta-oxidation pathways during microalgae conversion into methane. *Scenedesmus* spp. substrate and free ammonia concentration strongly shaped thermophilic reactor microbial structure. Partial Least Square Discriminant Analysis (PLS-DA) remarked the resilient role of minor groups related to *Thermogutta*, *Armatimonadetes* and *Ruminococcaceae* against a potential inhibitor like free ammonia. Towards low-cost biogas production from microalgae, this study reveals valuable information about thermophilic microorganisms that can strongly disrupt microalgae and remain in high solids retention anaerobic digesters.

Keywords

16S rRNA gene; anaerobic digestion; bioreactor; microalgae; microbial community; renewable energy

Publication

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5.1.Introduction

The composition of microalgae is heterogeneous among cultures, but it is commonly characterized by a high content in proteins and complex polysaccharides as cellulose-like layers (Baudelet *et al.*, 2017). Some *Chlorophyta* microalgae that can be used for nutrient removal like *Chlorella* and *Scenedesmus* genera have robust cell bodies (González-Fernández *et al.*, 2015). Thus, their application of *Chlorophyta* for bioenergy production through anaerobic digestion can become challenging as this process is limited by the hydrolysis stage of the substrate (Zamalloa *et al.*, 2012a). Several authors have efficiently overpassed this barrier through the application of pre-treatments. According to Ometto *et al.* (Ometto *et al.*, 2014), enzymatic approaches exhibit the greatest biogas yield increments, compared to thermal or ultrasonication pre-treatment methods. However, these interesting strategies might not be feasible when upscaling the technologies for microalgae conversion into energy. As an alternative, other studies focus on the natural enzymatic capacity of several microorganisms for breaking recalcitrant plant-based compounds (Shrestha *et al.*, 2017).

High biodegradation values of untreated microalgae had been previously reported from bioaugmentation processes with relevant cellulose degraders such as *Clostridium thermocellum* strain DSM 2360. The green microalgae *Chlorella vulgaris* was digested in a batch system with the consequent 24.0% increase of the methane yield (Lü *et al.*, 2013). A later study by Lavric and co-workers using the same strain achieved a 62.0% biodegradation of a microalgal mix from a high-rate algal pond, yet including a thermal pre-treating step for disruption (Lavrič *et al.*, 2017). These interesting strategies are microbial based and therefore promising but lack relevant information about their viability in continuous systems like high retention anaerobic digesters.

The evaluation of the long-term stability of selected microbes and their enzymes in an engineered system should be explored to assess the feasibility of microbial based strategies (Odnell *et al.*, 2016). According to this, a forward step for microalgae conversion into energy is required, *i.e.* new

approaches should consider both the influence of operational conditions over microbial communities and their viability over time. Parameters like the Sludge Retention Time (SRT) or the Organic Loading Rate (OLR) have a strong influence over the diversity of microbial communities (Kundu *et al.,* 2017; Vanwonterghem *et al.,* 2014b), and might affect the microalgae disruption efficiency. Acclimatized microbial communities must be not only suitable for raw microalgae disruption, but also resilient, tolerant or have functional redundancy to overcome process disturbances (Briones and Raskin, 2003). The main drawback of thermophilic digestion of protein enriched substrates such as microalgae is the inhibition by free ammonia (Sialve *et al.,* 2009). Several microbial groups likewise the methanogens are sensitive to this reduced form of nitrogen, decreasing biomethanization yields in thermophilic reactors. Nevertheless, little is known about the effect of this inhibitor over the potential hydrolytic microbes and other key groups involved in the microalgae disruption. Although raw microalgae anaerobic digestion has been widely reported in mesophilic studies including continuous reactors (González-Fernández *et al.,* 2015; Klassen *et al.,* 2016), but with very little information about their microbial community compositions (González-Fernández *et al.,* 2018; Klassen *et al.,* 2017; Sanz *et al.,* 2017) thermophilic systems are less used for this purpose. However, operation temperatures over 50ºC might have a positive effect over hydrolytic microorganisms and their enzymatic reactions.

The present study evaluates the long-term community established in a thermophilic reactor for microalgae degradation. A raw microalgal biomass feedstock coming from a photobioreactor pilot plant was digested at high constant SRT and different OLR values in a continuous system. A 16S rRNA gene analysis was performed along the 18-month experience revealing the composition of the biogas-producing thermophilic community for microalgae disruption established in the reactor.

5.2.Materials and Methods

5.2.1. Thermophilic reactor performance

Raw microalgae were continuously converted into biogas for 18-months in a thermophilic continuous stirred tank reactor of 1.6 L working volume (0.4 L headspace volume). The digester was inoculated with a thermophilic biomass coming from a pilot-scale digester (Valladolid, Spain). The biomass was mechanically stirred and maintained at 55ºC in the digester with an SRT and hydraulic retention time (HRT) of 50 days. The microalgae biomass was continuously harvested from a membrane photobioreactor pilot plant situated in the WTTP "Barranc del Carraixet" (Valencia, Spain) (Viruela *et al.,* 2016). The phototrophic culture was dominated by *Scenedesmus* spp. according to González-Camejo *et al.* (González-Camejo *et al.,* 2017). Microalgae were concentrated with a cross-flow ultrafiltration hollow-fiber system (5.7-11.7 $gVSS \cdot L^{-1}$). High free ammonia concentrations can be reached in anaerobic systems treating microalgae, especially under thermophilic conditions (Sialve *et al.,* 2009; Ward *et al.,* 2014). Thus, low OLR values (0.2, 0.3 and 0.4 $gCOD_{inf} \cdot L^{-1} \cdot d^{-1}$) were chosen in this study to avoid a process failure due to free ammonia inhibition, since protein degradation (microalgae common content ranges 6-52% (Sialve *et al.,* 2009)) can lead to free ammonia accumulation in the system. Three correspondent pseudo-steady state conditions were reached during each OLR scenario and characterized in terms of microbial population and main physicochemical parameters. It was considered that the reactor run under pseudo-steady state conditions when the process exhibited stability in terms of solid concentration and biogas production for at least four weeks ($n \geq 4$).

5.2.2. Nucleic material extraction and sequencing of 16S rRNA gene

This study is a long-term performance in a continuous reactor and therefore samples belonging to the same pseudo-steady state period are considered biological replicates of the thermophilic reactor microbial community. The samples were extracted from the reactor after 248, 268 and 276 days (samples T01, T02, T03; respectively from Period 1), 408, 422 and 443 days (samples T04, T05, T06; respectively from Period 2), 549 and 568

days (samples T07, T08; respectively from Period 3). Resulting pellets from 1 mL digestate samples were stored in 2 mL cryotubes at -20ºC. The E.Z.N.A DNA Extraction Kit for Soil (Omega-Biotek) was used for nucleic acid material extraction from 0.5 g biomass, according to the manufacturer's protocol. Resulting DNA was quantified in a fluorometric assay for dsDNA with Qubit 2.0 (Thermo Scientific).

16S rRNA gene analysis of *Bacteria* and *Archaea* microorganisms was performed through amplicon sequencing. Libraries were prepared using specific primers for the v3-4 hyper variable region of the target gene (341F 5'-CCTACGGGNGGCWGCAG-3' and 806R 5'-GGACTACNVGGGTWTCTAAT-3') (Takahashi *et al.*, 2014). The sequencing run was carried out in a 2x300 bp paired-end run using v3 chemistry in a MiSeq Sequencer (FISABIO, Valencia, Spain). Raw sequences were deposited on the NCBI database (BioProject PRJNA434206, SRP132920).

5.2.3. Illumina data processing

The resulting raw sequences were first trimmed and processed through the algorithm prinseq (Schmieder and Edwards, 2011), applying a quality threshold of 30. The merging of each forward and reverse read was performed within fastq-join (Aronesty, 2011) and checked for chimeras with UCHIME (Edgar *et al.*, 2011) using default parameters. The downstream analysis of the filtered and high-quality resulting sequences was performed in QIIME 1. A 3% dissimilarity value between sequences was chosen for open reference otu-picking. The resulting Operational Taxonomic Units ($OTU_{0.97}$) were taxonomically assigned with the 16S rRNA-based LTP 128 release of the SILVA database. Final data was normalized to the minimum number of filtered paired-end sequences obtained. Additionally, $OTU_{0.97}$ below 0.01% were removed to reduce biases, as Lê Cao *et al.* (Lê Cao *et al.*, 2016) proposed for statistical analysis of amplicon sequencing data. Those $OTU_{0.97}$ assigned to *Cyanobacteria/Chloroplast* were attributed to the feedstock and non-functional organelles or cell bodies and thus not considered for microbial ecology analysis of digester.

5.2.4. Microbial diversity analysis from 16S rRNA sequencing data and statistics

Biodiversity of the thermophilic community was evaluated through the estimator *Simpson* index, which accounts alpha diversity considering species richness. Beta diversity of the community was explored through Principal Co-ordinate Analysis of the weighted UniFrac distance matrix retrieved from QIIME. Partial Least Square-Discriminant Analysis (PLS-DA) was performed in R-Studio according to Lê Cao *et al.* (Lê Cao *et al.*, 2016) to analyze the free ammonia effect over the microbial community members. PLS-DA is a powerful statistical modeling approach that allow the interpretation of big data matrixes like those resulted from the thermophilic reactor microbial composition 16S rRNA gene analysis. This multivariate statistical technique is usually performed to sharpen and maximize the separation between groups of samples according to their covariance values. PLS-DA is very useful because provides invaluable insight into the causes of the discrimination through the weights and loadings of the constructed model. The most discriminant groups were extracted after PLS-DA according to their Variable Importance Parameter (VIP).

5.3.Results and discussion

5.3.1. Microalgae biomethanization during thermophilic reactor performance

Three stable periods defined by an OLR of 0.2, 0.3 and 0.4 $gCODinf \cdot L^{-1} \cdot d^{-1}$ were achieved in the reactor using raw microalgae biomass as the only feedstock. The parameters determined thrice weekly during the three different pseudo-steady state periods are summarized in Table 5.1, including the mean values and the standard deviation of CH_4 percentage content in biogas, methane yield (calculated as $mLCH_4.gCOD_{inf}^{-1}$), concentration of ammonia ($mgN\text{-}NH_4 \cdot L^{-1}$), free ammonia concentration ($mgN\text{-}NH_3 \cdot L^{-1}$), VFA concentration ($mgCH_3COOH \cdot L^{-1}$) and alkalinity (measured as $mgCaCO_3 \cdot L^{-1}$). The complete performance of this reactor is well reported in a recent work (Greses, 2017).

The anaerobic biodegradability of *Scenedesmus* biomass ranges 22-24% under mesophilic conditions, according to Gonzalez-Fernandez et al (González-Fernández *et al.*, 2012; Greses, 2017). A recent study (González-Fernández *et al.*, 2018) performed under thermophilic conditions using an acclimated inocula reached a methane yield of 108.2 mL $CH_4 \cdot gCOD_{influent}^{-1}$ from raw *Scenedesmus* biomass. Hence, the favorable effect of acclimation of the microbial population in this work is here suggested. Biodegradabilities around 32-41% were achieved in the thermophilic CSTR operated at high solids retention time. The higher acclimation of the biomass to the microalgae feedstock, the higher release of hydrolyzed compounds that can be finally turned out into methanogenic substrates. However, the low C:N ratio of this biomass might have an effect over the methanogenic activity. Indeed, the limited methane yield observed during Period 2 might be related to the presence of free ammonia forms that could inhibit the methanogenic population. Besides, the high concentration of the feedstock (5.7-11.7 gVSS·L-1) applied to maintain both HRT and SRT at 50 days could disturb the biological process.

Table 5.1. Characterization of the thermophilic reactor periods used for microbial analysis, mean values and standard deviation are shown (n≥4).

	Period 1	**Period 2**	**Period 3**
OLR ($g.d^{-1} \cdot L^{-1}$)	0.2	0.4	0.3
pH	7.14 ±0.02	7.37 ±0.05	7.40 ±0.06
CH_4 biogas content (%)	62.6 ±1.9	61.9 ±1.6	62.5 ±0.9
YCH_4 ($mLCH_4.gCODinf^{-1}$)	110.9 ±3.2	131.8 ±0.4	143.7 ±3.9
Biodegradability (%)*	32±1	38±1	41±1
NH_4^+ ($mgN\text{-}NH_4 \cdot L^{-1}$)	365.3 ±14.8	750.6 ±17.5	652.1 ±10.4
NH_3 ($mgN\text{-}NH_3 \cdot L^{-1}$)	19.7 ±0.8	71.7 ±8.8	62.2 ±3.1
VFA ($mgCH_3COOH \cdot L^{-1}$)	124.8 ±17.8	497.1 ±27.1	79.7 ±28.6
ALK ($mgCaCO_3 \cdot L^{-1}$)	1559.8 ±27.3	2333.1 ±62.3	2443.7 ±90.3

*Calculated considering a theoretical potential of 350 $mLCH_4 \cdot gCOD_{influent}^{-1}$ (Greses, 2017).

5.3.2. 16S rRNA sequencing data processing results.

The amount of raw sequences retrieved from the Illumina sequencing paired-end run ranged 86,719-40,669 joined reads per sample. After strict trimming step and singletons removal, the amount decreased to 71,138-33,436 sequences per sample. The alpha diversity analysis of each sample

reported valuable information about the number of species detected through open reference $OTU_{0.97}$ clustering analysis. A considerably high diversity in terms of species richness was elucidated from the more than 1,500 $OTU_{0.97}$ found during the whole experience (Table 5.2). Environmental samples are typically inhabited by complex communities characterized by a high number of observed species (Briones and Raskin, 2003). The number of observed species in the reactor ranged 1,445-2,621 and consequently a high Simpson index was observed between samples (0.86±0.03, n≥2). These values are among the range of values observed in similar studies e.g. thermophilic reactor treating a complex polysaccharide substrate, 0.72-0.98 Simpson index (Sun *et al.*, 2015).

Table 5.2. Amplicon sequencing approach related information and alpha diversity analysis among samples

	Period 1 (OLR*=0.2)			Period 2 (OLR=0.4)			Period 3 (OLR=0.3)	
	T01	**T02**	**T03**	**T04**	**T05**	**T06**	**T07**	**T08**
Raw	44,692	46,166	86,719	52,279	43,825	85,324	65,762	77,723
Joined/Filtered	37,059	38,344	64,863	39,045	33,436**	71,138	57,306	63,203
Observed $OTU_{0.97}$	1,980	1,677	2,621	1,445	1,530	2,171	1,789	2,190
Simpson	0.89	0.91	0.89	0.84	0.82	0.86	0.84	0.85

*OLR is expressed as $g.d^{-1}.L^{-1}$. **Minimum value of filtered sequences used for normalization of the dataset.

Compared to an extended diversity survey over thermophilic and mesophilic full-scale digesters based on pyrosequencing of the 16S rRNA gene (Sundberg *et al.*, 2013), the diversity characterizing the thermophilic microalgae reactor is high in terms of observed OTUs. This diversity was taxonomically assigned to 50 phyla, 139 classes, 232 orders, 410 families and 823 genera from both *Bacteria* and *Archaea* domains. The microbial community found in the reactor was mainly assigned to six dominant *Bacteria* phyla (*Thermotogae*, *Firmicutes*, *Atribacteria*, *Aminicenantes*, *Synergistetes* and *Planctomycetes*) and two *Archaea* orders (*Methanobacteriales* and *Methanosarcinales*), *including a Methanosaeta* related $OTU_{0.97}$ detected by the open clustering approach. These phylotypes were dominant in the reactor and represented the 77.3±3.9% of the cumulative community relative abundance (see Table 5.3). According to several authors, high diversity in anaerobic digesters is linked to a good

performance (Kundu *et al.*, 2017). However, it should be remarked that biodiversity *per se* cannot guarantee the stability of anaerobic systems. Ecological aspects such as functional redundancy, resilience and resistance (Allison and Martiny, 2008) of a certain community should be evaluated to help us to improve process stability in anaerobic reactors though a deeper comprehension of microbial community composition.

5.3.3. A long-term microbial community characterization of thermophilic anaerobic digestion of microalgae in a continuous system

The microalgae mixed culture harvested from the photobioreactor pilot plant was mainly composed of *Scenedesmus* spp., which are characterized by a thick cellulosic material layer and a recalcitrant biopolymer *i.e. algaenan*, plus a mixture of neutral sugars, proteins and uronic acid (Baudelet *et al.*, 2017). Thus, this *Chlorophyta* microalgae can trigger different degradation anaerobic pathways suitable for methane production. For microbial ecology interpretation, only methanogenic *Archaea* and those dominant *Bacteria* phylotypes defined at genus level with relative abundance values over 2.0% were selected. Figure 5.1 shows the composition of the different phylotypes found in each sample during the microalgae thermophilic digestion.

The significant content of proteins of *Scenedesmus* microalgae (Baudelet *et al.*, 2017; Ward *et al.*, 2014) makes peptidic hydrolytic and fermentative pathways during anaerobic digestion critical. *Coprothermobacter* was among the dominant phylotypes observed in the reactor. The overwhelming potential of *Coprothermobacter* related microorganisms for protein degradation is well known and has been extendedly reviewed (Gagliano *et al.*, 2015). Its relative abundance in the thermophilic reactor ranged among 15.1-35.6% during the continuous operation. These results suggest the implication of this phylotype in the first stage of the microalgae disruption by releasing peptidases that can degrade *Scenedesmus* cells.

Table 5.3. Relative abundance values of dominant phylotypes detected in the thermophilic reactor.

			Period 1 (OLR = 0.2)			Period 2 (OLR = 0.4)			Period 3 (OLR = 0.3)		
SILVA Accession No.	***Role****	***Ref.***	***T01***	***T02***	***T03***	***T04***	***T05***	***T06***	***T07***	***T08***	***Taxonomic Classification*****
EF515700.1.1413	PF	(Kuroda et al., 2016)	4.8	7.0	6.0	0.0	0.0	0.0	0.2	0.3	*d.-Bacteria; p.-Aminicenantes*
EF586052.1.1457	SF	(Dodsworth et al., 2014)	2.6	1.6	1.8	2.4	2.7	2.5	3.4	2.9	*d.-Bacteria; p.-Atribacteria; c.-Atribacteria Incertae Sedis; o.-Unknown Order; f.-Unknown Family; g.-Candidatus Caldatribacterium*
CP001251.796084.797624	H,SF	(Brumm et al., 2016)	2.2	3.9	3.0	6.9	4.9	4.0	3.8	4.4	*d.-Bacteria; p.-Dictyoglomi; c.-Dictyoglomia; o.-Dictyoglomales; f.-Dictyoglomaceae; g.-Dictyoglomus spp.*
FJ769444.1.1396	H,PF	(Gagliano et al., 2015)	35.6	32.8	33.0	19.7	18.4	15.1	19.7	14.9	*d.-Bacteria; p.-Firmicutes; c.-Clostridia; o.-Thermoanaerobacterales; f.-Thermodesulfobiaceae; g.-Coprothermobacter sp.*
FN436058.1.1503	SF	(Hagen et al., 2017a)	3.4	3.6	3.7	1.5	0.9	1.5	2.0	2.8	*d.-Bacteria; p.-Planctomycetes; c.-Planctomycetacia; o.-Planctomycetales; f.-Planctomycetaceae; g.-Thermogutta sp.*
EF559055.1.1480	PF	[27]	9.3	9.5	9.0	6.7	4.2	5.9	7.8	6.1	*d.-Bacteria; p.-Synergistetes; c.-Synergistia; o.-Synergistales; f.-Synergistaceae; g.-Anaerobaculum sp.*
AY862527.1.1347	-	***	8.0	2.8	11.7	37.6	40.7	36.4	39.7	38.9	*d.-Bacteria; p.-Thermotogae; c.-Thermotogae; o.-EM3*
CU919211.1.1345	PF	(Maus et al., 2016)	4.7	4.1	2.8	2.9	3.1	2.3	2.0	2.6	*d.-Bacteria; p.-Thermotogae; c.-Thermotogae; o.-Petrotogales; f.-Petrotogaceae; g.-Defluviitoga sp.*
EU638683.1.1349	H,SF	(Shrestha et al., 2017)	2.0	8.0	3.9	3.3	3.0	4.5	2.2	5.6	*d.-Bacteria; p.-Thermotogae; c.-Thermotogae; o.-Thermotogales; f.-Fervidobacteriaceae; g.-Fervidobacterium sp.*
DQ785500.1.922	HM	(Maus et al., 2016; Sundberg et al., 2013)	0.2	0.1	0.2	0.5	0.3	0.1	0.2	0.2	*d.-Archaea; p.-Euryarchaeota; c.-Methanobacteria; o.-Methanobacteriales; f.-Methanobacteriaceae*
FN646546.1.939	HM	(Maus et al., 2016; Sundberg et al., 2013)	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0	*d.-Archaea; p.-Euryarchaeota; c.-Methanobacteria; o.-Methanobacteriales; f.-Methanobacteriaceae; g.- Methanothermobacter sp.*
New ref. OTU#204	AM	(Maus et al., 2016; Sundberg et al., 2013)	0.5	0.8	0.7	1.4	0.6	0.3	0.5	0.4	*d.-Archaea; p.-Euryarchaeota; c.-Methanomicrobia; o.-Methanosarcinales; f.-Methanosaetaceae; g.-Methanosaeta sp.*
Cumulative relative abundance (%)			73.5	74.2	75.9	83.0	78.9	72.7	81.6	79.0	

*Role and reference used for ecological interpretation, H: hydrolytic; SF: saccharolytic fermenter; PF: peptidic fermenter; B: 2F: second fermenter; HM: hydrogenotrophic methanogen; AM: acetoclastic methanogen. **Taxonomic levels have been abbreviated from domain (d.) to genus (g.) level.***Hatamoto *et al.*, 2007; Oosterkamp *et al.*, 2016; Thiel *et al.*, 2016.

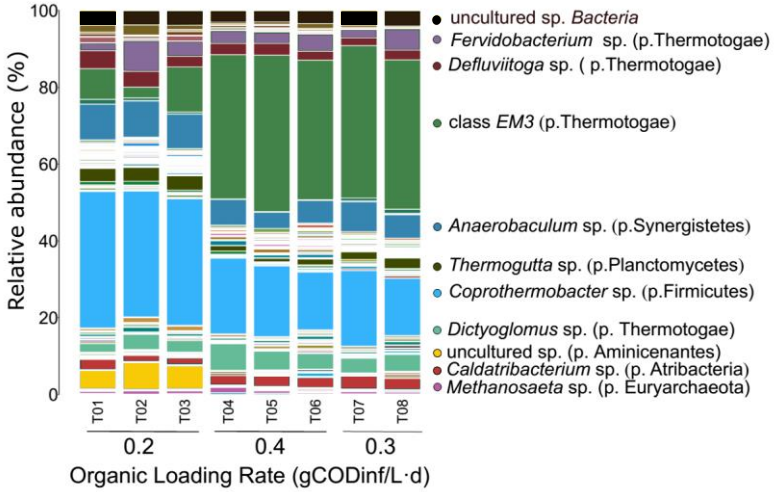

Figure 5.1. Relevant phylotypes classified at the lowest known taxonomic level among the three periods studied in the thermophilic reactor. White color has been used for phylotypes below 2% relative abundance in every sample to facilitate the visualization of the barplots. Samples belonging to the same pseudo-steady state period are biological replicates of reactor biomass

After the disruption of proteins, amino acid fermenters like *Aminicenantes* and *Anaerobaculum* phylotypes can easily take the released peptides. No cultured strain has been related up to date inside the *Aminicenantes* phylum. However, as Kuroda and co-workers recently reported (Kuroda *et al.*, 2016), amino acid fermentation might be their main implication in anaerobic digesters. *Anaerobaculum* related microorganisms have been previously observed in thermophilic full-scale digesters treating plant-derived substances (Weiss *et al.*, 2008). These authors also suggested the peptidic fermentation capacity of these microorganisms. Other fermenters detected in the present study such as *Caldatribacterium*, *Thermogutta* and *Fervidobacterium*, have been associated on the other hand to saccharolytic pathways (Dodsworth *et al.*, 2014; Gagliano *et al.*, 2015; Kuroda *et al.*, 2016). The relevance of these groups in anaerobic digesters relies on their implication as methanogenic precursor producers, as acetate, carbon dioxide and hydrogen are among their fermentative products.

The remarkable relative abundance of hydrolytic and fermentative phylotypes in the thermophilic reactor was attributed to the capacity of the digester biomass for microalgae degradation. The presence of the phylotypes *Dictyoglomus, Defluviitoga* besides the abovementioned *Caldatribacterium*, *Thermogutta* and *Fervidobacterium* has been reported in studies where recalcitrant substrates were treated at high operational temperature. Several glycosyl hydrolases were found in a recent metagenomic study that revealed the important role of *Dictyoglomus* genus in the fermentation of plant-based carbohydrates (Brumm *et al.*, 2016). The metatranscriptomic analysis of a full-scale thermophilic plant treating agricultural wastes highlighted the role of *Defluviitoga* for breaking hemi-celluloses (Maus *et al.*, 2016), which are some of the main constituents of *Scenedesmus* cell walls. The authors found high relative activity values of *Defluviitoga* and suggested their role as main saccharolytic fermenters. A recent multi-omic study supports the relevance of the *Thermogutta* phylotype during fermentation stages in thermophilic digesters (Hagen *et al.*, 2017). Little is known about the *Atribacteria* phylum and one of the phylotypes identified in the present study: *Caldatribacterium*. According to a recent metagenomic reconstruction from different environmental samples including anaerobic digesters (Nobu *et al.*, 2016), this phylotype is a potential carbohydrate fermenter. In this thermophilic microalgae digester, the higher hydrolysis of microalgae cell walls, the higher release of cellulose- and hemicellulose- derived monomers that might trigger the presence of saccharolytic fermenters. Finally, the remarkable presence of the EM3 group in the reactor must be separately discussed as its metabolic roles remain poorly characterized (to the knowledge of the authors of the present manuscript).

This is the first study of microbial dynamics in an anaerobic digester where high relative abundance values of EM3 group have been continuously observed. This group was recently affiliated to the *Thermotoga* phyla after metagenomic analysis of the anoxic under layer of phototrophic microbial mats (Thiel *et al.*, 2016). According to these authors, EM3 related microorganisms could be involved in fermentation pathways, providing

hydrogen to other members of the microbial community. Nevertheless, other previously reported studies suggested that the metabolic implications of EM3 in a thermophilic digester could be more diverse, as its presence has been detected in a similar system treating lignocellulose although in lower relative abundances compared to the present study (Oosterkamp *et al.*, 2016). On the other hand, a previous study based on RNA stable isotope probing identified EM3-related microbial groups during the thermophilic conversion of long-chain fatty acids into methane (Hatamoto *et al.*, 2007). Hydrogen is commonly released during fermentation of hydrolyzed microalgae components under thermophilic conditions. Also, beta oxidation of long-chain fatty acids should be considered when treating microalgae grown in a pilot plant, as stress conditions boost the lipid intracellular accumulation in the microalgae bodies (Chen *et al.*, 2017). Hence, suggested metabolic roles for EM3 group in this study would be: (i) disruption of microalgae cell walls, (ii) uptake of substrates released after microalgae hydrolysis such as carbohydrate monomers or (iii) beta oxidation of intracellular lipids. Further research with a deeper comprehension of the metabolic pathways of EM3 could help to elucidate the role of this group in degradation of *Scenedesmus* biomass.

Summarizing, the coexistence of potential proteolytic phylotypes such as *Coprothermobacter*, jointly with scavengers of amino acids like *Anaerobaculum*; besides the suitable hydrolytic and saccharolytic role of *Fervidobacterium*, *Dictyoglomi*, and *Defluviitoga* phylotypes and the potential implication of EM3 in other relevant pathways (hydrolysis, hydrogen-producing fermentation or beta-oxidation), might allow the disruption of untreated microalgae into different by-products that can be further turned into methane by methanogenic microorganisms. Furthermore, the syntrophic relationships among these dominant microbes might have also allowed the thermophilic reactor to host a fast transference of the even more reduced compounds released from the digested microalgae cell bodies. The presence of these relevant phylotypes over time in the reactor operated

at high SRT (50 days) suggests their convenient use for continuous degradation of raw microalgae.

5.3.4. From microalgae to biogas: relevance of acetoclastic methanogens and free ammonia.

An appropriate environment for methane production was promoted in the reactor since microalgae degradation releases methane precursors such as acetate as well as carbon dioxide and hydrogen, giving rise to the methane productivities shown in Table 5.1. In the present study, according to the relative abundances observed of methanogenic phylotypes, the dominant group were *Methanosaeta* spp. (Table 5.3). The acetoclastic pathway is well reported for this group of methanogens (Maus *et al.*, 2016; Sundberg *et al.*, 2013) and several authors have suggested its acclimatization capacity to free ammonia for enhancing the digester performance in terms of stability and methane production when degrading microalgae (Mahdy *et al.*, 2017). However, this group dramatically decreased during Period 2 from 1.4% to 0.3% (Table 5.3). It should be remarked that the highest concentration of *Scenedesmus* was fed to the reactor during this period, where also a VFA accumulation of 491.1 $mgCH_3COOH \cdot L^{-1}$ was determined. These results suggest that the highest hydrolytic activity of the thermophilic reactor was achieved in Period 2 but did not result in highest biomethanization values due to a partial inhibition of the methanogenic pathways.

Only minor groups of hydrogenotrophic methanogens belonging to *Methanobacteriaceae* were observed besides *Methanosaeta* in the thermophilic reactor, suggesting that the acetoclastic pathways were dominant in the reactor. The relative abundance of these methanogens increased 2-fold during Period 2, where the maximum OLR of *Scenedesmus* was fed to the reactor. As abovementioned, this period was characterized by a VFA accumulation and suggested as a partially inhibited methanogenic state. Hydrogen scavenging by *Methanobacteriaceae* has a relevant role in thermophilic digesters or when acetoclastic methanogens are inhibited, providing robustness and resilience to the process (Hagen *et al.*, 2017). In the same logic, the microalgae digester might recover its methanogenic

capacity in Period 3 after enhancing other methanogenic pathways different to the acetoclastic, as an absence of VFA accumulation and the highest biomethanization values 143.7±3.9 $mLCH_4 \cdot gCOD_{inf}^{-1}$ were finally observed in Period 3. In this period, *Methanosaeta* remained as dominant methanogens although at lower relative abundances (0.4±0.1%) than in previous periods. These findings suggest a slight acclimatization capacity of *Methanosaeta* to the free ammonia values achieved in the thermophilic digester (62.23±3.09 $mgN\text{-}NH_3 \cdot L^{-1}$).

5.3.5. Microalgae feedstock overdrives microbial community structure in the thermophilic reactor

The disruption of the microalgae cell bodies was carried out by the presumably hydrolytic population enriched during long-term operation of the reactor. According to different studies feedstock has a strong influence over the microbial community structure of anaerobic reactors [10,11]. In the present study, the characteristics of *Scenedesmus* biomass might shape the microbial community over-time. This hypothesis was explored by weighted UniFrac phylogenetic distances calculation between samples and later principal component analysis of the resulting matrix (see the Principal Co-ordinates Analysis (PCoA) resulting plot in Figure 5.2).

The closest distance between samples, the higher similarity among them according to their $OTU_{0.97}$ composition and their relative abundances, which are considered for UniFrac distances calculation. The shift in the microbial community structure took place when the OLR was increased from 0.2 to 0.4 $gCOD_{inf} \cdot L^{-1} \cdot d^{-1}$ (Period 1 and 2, correspondingly). This variability is provided by the first component extracted from the PCoA that explains the 88% of the differences in between samples. The microbial community change was irreversible despite of decreasing the OLR to 0.3 $gCOD_{inf} \cdot L^{-1} \cdot d^{-1}$ (Period 3). These results suggest the acclimatization of the community to a very specific substrate that achieved a 143.7 $mLCH_4.gCOD_{inf}$ during thermophilic anaerobic degradation of *Scenedesmus* biomass.

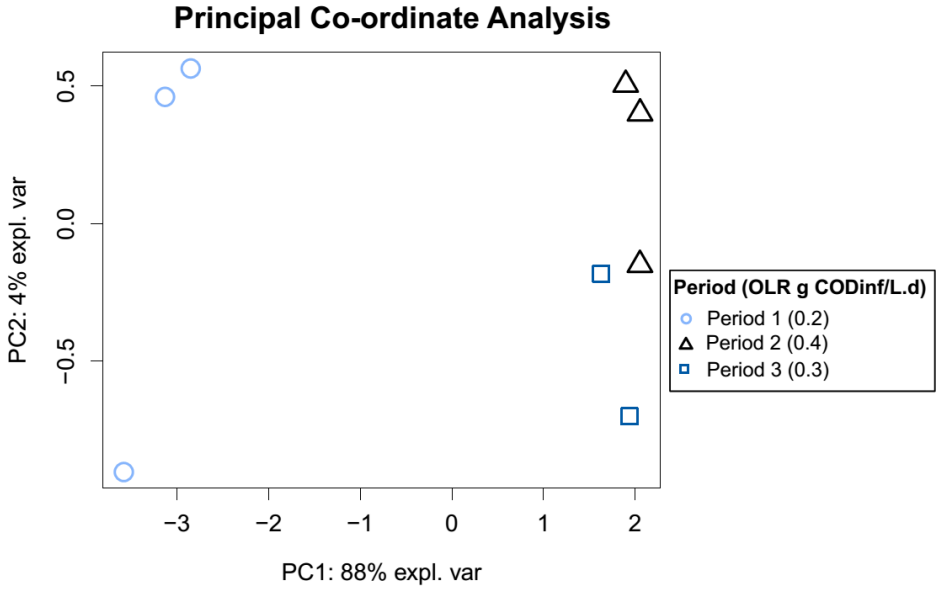

Figure 5.2. Principal Co-ordinates Analysis (PCoA) of weighted UniFrac thermophilic $OTU_{0.97}$ distance matrix. Explained variance by each component is indicated in each axis in percentages.

The resilience of the community established during Period 2 can be suggested from the results observed in Figure 5.2. As shown in Table 5.1, a partial inhibition of the methanogenic members of the thermophilic reactor was elucidated from the consequent VFA accumulation in the reactor. However, the second component extracted from the PCoA has a very low explanatory value of 4%. Although the main difference in between Period 2 and 3 was the presence of absence of VFA accumulation due to the free ammonia values achieved, the microbial community structure of the thermophilic reactor remained stable. Hence, the presumably hydrolytic bacteria members found in the present study for *Scenedesmus* disruption in absence of other pre-treatments would have a certain tolerance to free ammonia concentrations that on the other hand affected the activity of other microorganisms like *Methanosaeta*.

5.3.6. PLS-DA reveals resilient non-dominant phylotypes involved in microalgae thermophilic degradation.

The shift in the microbial diversity observed during VFA accumulation after partial inhibition by free ammonia released in the reactor has been thoroughly discussed in this study through analysis of the most abundant phylotypes. However, less abundant groups might also have a relevant response in the digester to operational disturbances. From an ecological perspective, the presence of minor groups can provide resilience to a certain environment when they are functionally redundant as different microorganisms inhabiting the same niche can be involved in similar metabolic pathways but have different phylogenetic or physiological characteristics (Lynch and Neufeld, 2015).

PLS-DA analysis was applied to the microbial community composition discriminating between the presence of potential inhibitors like free ammonia observed at low (Periods 1 and 3) or high (Period 2) concentration in the thermophilic reactor. This inhibitor can act as selective factor decreasing the diversity of the system, but it might also enhance the resilience of the biomass, as some of the key microorganisms might have acclimatization potential to inhibitors. As can be seen in Figure 5.3, the developed PLS-DA model extracted for free ammonia perfectly separates the observations from low and high inhibition levels reached in the reactor.

The resulting PLS-DA plot shown in Figure 5.3 was constructed from the first and the second components extracted from the analysis, which showed up the highest variability values. The first component explained a 19% of the covariance among samples, whereas the second component denoted a 13% (related to the feedstock influence as previously discussed in this manuscript). To reveal the most discriminant microbial groups fitting the model, the Variable Importance in the Projection coefficient (VIP) was extracted from the PLS-DA. VIP values are indicators of the explanatory power of each $OTU_{0.97}$ over the predicted variate (high level inhibitors, in this case). The discriminant $OTU_{0.97}$ values were decreasingly sorted

according to their VIP value. The 30 top $OTU_{0.97}$ groups are shown in Table 5.4 in alphabetical order of their taxonomy for ecological interpretation.

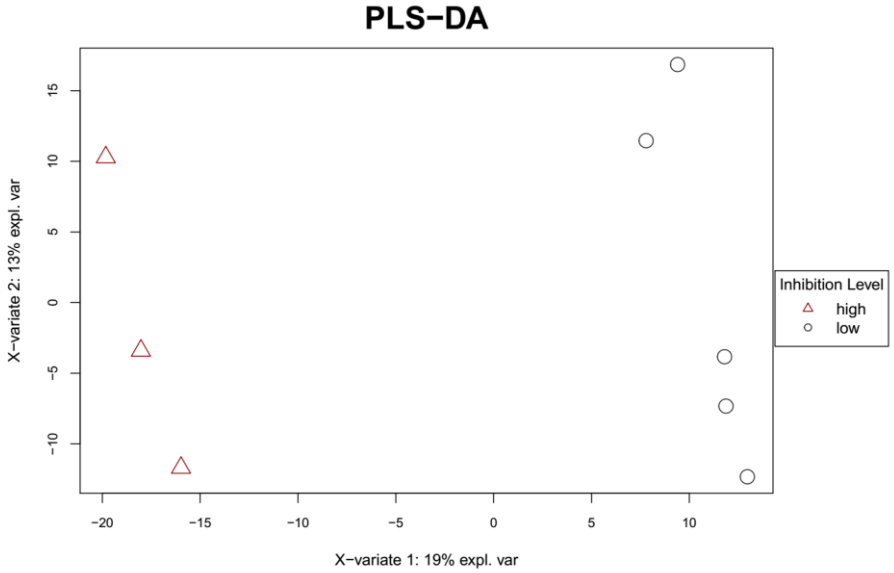

Figure 5.3. Evaluation of the high and low inhibitor level effect over reactor samples through Partial Least Squares Discriminant Analysis (PLS-DA). Samples with high levels of inhibitors (Period 2) are plotted with red triangles and low levels (Periods 1 and 3) with circle points.

Phylotypes affiliated to *Ruminococcaceae*, *Thermogutta* and *Armatimonadetes* were among the most discriminant (Table 5.4) during the accumulation of VFA in the digester in Period 2 because of the partial inhibition by free ammonia (see Table 5.1). Microorganisms belonging to the *Ruminococcaceae* family have been found during fermentation and biohydrogenation of *Scenedesmus* biomass (Lai *et al.*, 2016). Besides, a recent multi-omic study supported the relevance of the *Thermogutta* genus during fermentation stages in thermophilic digesters despite their common low abundance (Hagen *et al.*, 2017). Finally, related groups to the *Armatimonadetes* phylum contain several species with a strong capacity for branched or amorphous polysaccharide disruption such as *Chthonomonas* (Lee *et al.*, 2011). Their prevalence in plant-fed digesters was suggested by

Dunfield and co-workers (2012). Due to the lack of cultured strains from this phylum, it is still poorly characterized. Despite the low abundance of these minor phylotypes (as their relative abundances were below 2% and therefore not shown in Figure 5.1), their high explanatory power in the PLS-DA model extracted from the thermophilic 16S rRNA gene analysis suggests their relevance in the efficient conversion of raw microalgae into biogas in a continuous system. These results are in accordance with the hypothesis of functional redundancy in anaerobic digesters which has been suggested as an ecological strategy that ensures a reservoir of responses against different disturbances over time and thus stabilizing the system performance (Briones and Raskin, 2003).

According to the results here presented, the disruption of microalgae by different dominant and minor bacteria groups with functional redundancy for *Scenedesmus* disruption allow the thermophilic conversion of this substrate into methane over time in a continuous system. Long-term acclimatization of methanogens to the free ammonia that is commonly released from a low C:N substrate like *Scenedesmus* should be considered in future studies as a microbial-based strategy to guarantee the performance of a biological thermophilic anaerobic conversion of these microalgae. Also, the inhibition by free ammonia observed in the present study could be mitigated increasing the low C:N ratio of the influent to balance the protein content of microalgae. As a future step forward, the addition of complementary substrates with high carbon content could be explored to benefit from the hydrolytic potential of the acclimated community without disturbing the methanogenic population. Moreover, since the addition of a co-substrate would avoid the risk of free ammonia inhibition it will be possible to evaluate the feasibility of applying of this process at pilot or industrial scale, since full-scale digesters are operated at higher OLR values than the range chosen in the present work. Anaerobic co-digestion should be preferably explored in lab-scale anaerobic reactors where hydraulic and solids retention time can be decoupled and optimized, like the anaerobic membrane bioreactor (AnMBR) or the up-flow anaerobic sludge blanket

(UASB) systems. In this manner, different hydraulic retention times could be evaluated, also facilitating the acclimation of methanogens to the final products of microalgae protein degradation. Finally, it is worth highlighting that the combination of microalgae and other substrates resulting from municipalities (like food waste or sewage sludge streams) for bioenergy generation would be an attractive practice that would also meet with the basics of a circular economy perspective.

Table 5.4. Variable Importance in the Projection (VIP) of each $OTU_{0.97}$ from PLS-DA analysis.

VIP range	Phylum	Class	Order	Family	Genus
2.09-2.12	*Actinobacteria*	*Actinobacteria*	*Micrococcales*	*Demequinaceae*	*Lysinimicrobium*
			PeM15		
2.24	*Armatimonadetes*				
2.15-2.28	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*
		Sphingobacteriia	*Sphingobacteriales*	*PHOS-HE51*	
2.04-2.13	*Chloroflexi*	*Caldilineae*	*Caldilineales*	*Caldilineaceae*	
2.08-2.28	*Firmicutes*	*Bacilli*	*Bacillales*	*Planococcaceae*	*Planomicrobium*
		Clostridia	*Clostridiales*	*Clostridiaceae 1*	*Clostridium sensu stricto 13*
				Peptostreptococcaceae	*Proteocatella*
				Ruminococcaceae	*Ruminococcaceae UCG-010*
		Negativicutes	*Selenomonadales*		
2.04-2.07	*Planctomycetes*	*Phycisphaerae*	*Tepidisphaerales*	*Tepidisphaeraceae*	
		Planctomycetacia	*Planctomycetales*	*Planctomycetaceae*	*Pir4 lineage*
					Thermogutta
2.08-2.31	*Proteobacteria*	*Alphaproteobacteria*	*Caulobacterales*	*Hyphomonadaceae*	*Woodsholea*
			Rhizobiales	*Methylobacteriaceae*	*Meganema*
			Rhodobacterales	*Rhodobacteraceae*	*Rhodobacter*
				Rhodospirillaceae	
			Rickettsiales	*Rickettsiales*	*Candidatus Odyssella*
			Sphingomonadales	*Sphingomonadaceae*	*Sphingobium*
		Betaproteobacteria	*Burkholderiales*	*Alcaligenaceae*	
				Comamonadaceae	*Comamonas*
		Deltaproteobacteria	*Bdellovibrionales*	*Bacteriovoracaceae*	*Peredibacter*
		Gammaproteobacteria	*Thiotrichales*	*Thiotrichaceae*	*Thiothrix*
2.10	*Spirochaetae*	*Spirochaetes*	*Spirochaetales*	*Leptospiraceae*	
2.04	*Synergistetes*	*Synergistia*	*Synergistales*	*Synergistaceae*	
2.29	*Verrucomicrobia*	*Verrucomicrobiae*	*Verrucomicrobiales*	*Verrucomicrobiaceae*	*Roseimicrobium*

Conclusions

The long-term continuous study here performed has revealed valuable information about resilient and functionally redundant groups that can be jointly used to convert *Scenedesmus* into methane. Well-known genera like *Coprothermobacter*, *Defluviitoga*, *Fervidobacterium*, or *Dictyoglomi* and others that are poorly described such as the EM3 *Thermotogae* group (40% of the relative abundance values) were key groups during thermophilic anaerobic digestion of *Scenedesmus.* The resilience of the community against free ammonia remarkable presence was linked to the minor members

of *Thermogutta*, *Armatimonadetes* and *Ruminococcaceae*. The present study extends our knowledge of microbial communities and allows the selection of future microbial groups that can be applied during biological conversion of complex microalgae in conventional systems.

Author Contributions

NZL: conception and design, analysis and interpretation of the data, drafting of the article, collection and assembly of data. SG: start-up, operating and monitoring of thermophilic reactor and critical revision of the article for important intellectual content. DA and LB: statistical expertise, critical revision of the article for important intellectual content, analysis and interpretation of the data. AS: critical revision of the article for important intellectual content, provision of study materials or patients and obtaining of funding. All authors: final approval of the article

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Statements regarding conflicts, consent and human/animal rights

No conflicts, informed consent, human or animal rights applicable.

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6. Acclimatised rumen culture for raw microalgae conversion into biogas: linking microbial community structure and operational parameters in Anaerobic Membrane Bioreactors (AnMBR)

6. Acclimatised rumen culture for raw microalgae conversion into biogas: linking microbial community structure and operational parameters in Anaerobic Membrane Bioreactors (AnMBR)

Abstract

Ruminal fluid was inoculated in an Anaerobic Membrane Reactor (AnMBR) to produce biogas from raw *Scenedesmus.* This work explores the microbial ecology of the system during stable operation at different solids retention times (SRT). The 16S rRNA amplicon analysis revealed that the acclimatised community was mainly composed of *Anaerolineaceae, Spirochaetaceae, Lentimicrobiaceae* and *Cloacimonetes* fermentative and hydrolytic members. Overall, the dominance of *Fervidobacterium* and *Methanosaeta* was attributed to the highest biodegradability achieved in the AnMBR (62%). Different microbial community clusters were observed at different SRT conditions. Interestingly, syntrophic bacteria *Gelria* and *Smithella* were enhanced after increasing 2-fold the organic loading rate (OLR) suggesting their importance in continuous systems producing biogas from raw microalgae.

Keywords

anaerobic membrane bioreactor (AnMBR); biogas; microalgae; 16S rRNA gene; rumen

Publication

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6.1.Introduction

Natural environments like the stomach cavity of the ruminant living beings are interesting sources of hydrolytic microorganisms (Weimer *et al.*, 2009). Nowadays, methodologies for high-throughput sequencing analysis of rumen (McGovern *et al.,* 2018) are allowing to elucidate the ruminal fluid composition of different sources (Li *et al.,* 2019; Trabi *et al.,* 2019). Application of these microbial communities to complex feedstock conversion into valuable products, including biogas, has attracted the interest of the scientific community since 1980s (Gijzen, 2002). On the basis of a circular economy perspective, microalgae can be included as a post-stage of anaerobic treatment of sewage, being later harvested and finally used as a substrate to produce biogas (González-Fernández *et al.,* 2015; Stiles *et al.,* 2018). Moreover, their carbon dioxide biofixation capacity from the atmosphere can reduce the carbon footprint of future water resource recovery facilities integrating microalgae processes (Seco *et al.,* 2018a).

Some of the most common microalgae harvested from sewage and other water bodies have robust cell walls, like *Chlorophyta* belonging genera. Hemicelluloses and celluloses are needed to achieve high disruption values of recalcitrant microalgae (González-Fernández *et al.,* 2012; Mussgnug *et al.,* 2010). Cell walls from *Scenedesmus* (phylum *Chlorophyta*) also contain a recalcitrant and aliphatic compound, algaenan (Baudelet *et al.,* 2017), which difficult their disruption and further conversion into biogas. As a consequence, bioenergy production from microalgae via anaerobic digestion becomes challenging, with an intrinsic energetic and economic cost associated with their disruption (González-Fernández *et al.,* 2015). Several efficient physicochemical pretreatment strategies for microalgae biomass breakdown have been explored and evaluated during the last years (Passos *et al.,* 2015). However, they might not be feasible when operating at industrial scale and thus, alternative strategies with lower energetic demands need to be searched.

Biological strategies have been proposed for microalgae disruption, including commercial enzymatic mixtures, bioaugmentation with hydrolitic cultures and the use of natural hydrolytic consortia (Carrillo-Reyes *et al.*, 2016). Weimer *et al.* (2009) suggested the potential use of ruminal fluids to convert in a single bioengineered system, complex plant-based substrates into high value products like fatty acids or methane. Interestingly, Zhao *et al.* (2016) evaluated the efficiency of a batch system inoculated with cow rumen bacteria for microalgal disruption and found high rates of carboxylic acids production. In terms of biomethanization, another study determined a 58.0% efficiency when co-inoculating rumen from a slaughterhouse with anaerobic sludge to transform lignocellulosic substrates in methane (Deng *et al.*, 2018). Moreover, Barragan-Trinidad *et al.* (2017) reported a methane production of 193 mL $CH_4 \cdot gCOD^{-1}$ in a batch system inoculated with rumen and fed with *Scenedesmus* biomass. Several authors pointed out that adhesion capacity of ruminant-living microorganisms to the plant fibers is crucial for their disruption. Interstingly, high solids retention systems can potentially simulate this environment (Weimer *et al.*, 2009). Indeed, a high retention upflow anaerobic sludge blanket (UASB) has been reported for complex polysaccharide anaerobic digestion (Zhao *et al.*, 2016), as well as anaerobic sequencing batch reactors (Barnes and Keller, 2004) and microalgae digestion at high SRT in AnMBR (Greses *et al.*, 2017).

Some of the studies focused on the use of rumen in anaerobic digesters have partially explored the microbial community developed and determined the relevance of *Firmicutes*, *Bacteroidetes* or *Proteobacteria*, which are also among the common phyla of anaerobic digesters (McIlroy *et al.*, 2017). Recently, Deng and co-workers (2018) have evaluated a semi-continuous system co-inoculated with rumen and linked the presence of *Bacteroidales*, *Prevotellaceae* and *Rickenellaceae* to a 58% efficiency in terms of methane production. However, this yield decreased after an overload of lignocellulosic material that disturbed the hydrogenotrophic methanogens population. Therefore, further research is needed prior to up-scale a process for biological disruption of microalgae using ruminant sources. Besides the

organic loading rate (OLR) (Deng *et al.,* 2018) or the temperature (González-Fernández *et al.,* 2018), the effect over microbial population of essential operational parameters like solids or hydraulic retention times (SRT and HRT, respectively) remain poorly explored.

To the knowledge of the authors of the present manuscript, this is the first work revealing the 16S rRNA microbial community of a rumen AnMBR system and its associated long-term response to changes in operational conditions. This work has been performed for more than 14 months in a continuous AnMBR operated at high SRT and different OLR, feeding raw microalgae harvested from an outdoor photobioreactor pilot plant. Microbial ecology and bioengineering concepts are here combined to broad our knowledge on complex feedstock degradation through anaerobic digestion using membrane technology and natural hydrolytic communities *i.e.* a rumen inoculum.

6.2.Materials and methods.

6.2.1. Anaerobic system configuration and experimental design

Ruminal fluid extracted from a fistulated goat was used as an inoculum source for the anaerobic system. The extracted fluid was directly inoculated into the reactor after removal of coarse material through gauze straining. The volatile solids content in the ruminal fluid had a mass ratio of 0.75 volatile solids per total solids (VS/TS). The system worked at 39°C during more than 14 months since this temperature is close to the optimum for ruminal sources (Giménez *et al.,* 2017) and far from unfavorable conditions for mesophilic microorganisms.

The system was composed of two tanks with the same volume of 14 L (4 L of head-space volume). Tank 1 was used as the main tank of the system whereas Tank 2 was simply used as a continuous biomass reservoir. This second tank was added to the system after 28 days of operation and it was neither considered for process performance nor to study any operational parameter. The digestate extracted from Tank 1 to control the SRT of the system was thus stored in Tank 2 to preserve a biomass that could be later

used as a new inoculum or reintroduced in Tank 1 after a performance failure, for example. A detailed diagram of the anaerobic system can be found in Figures 6.1a, 6.1b.

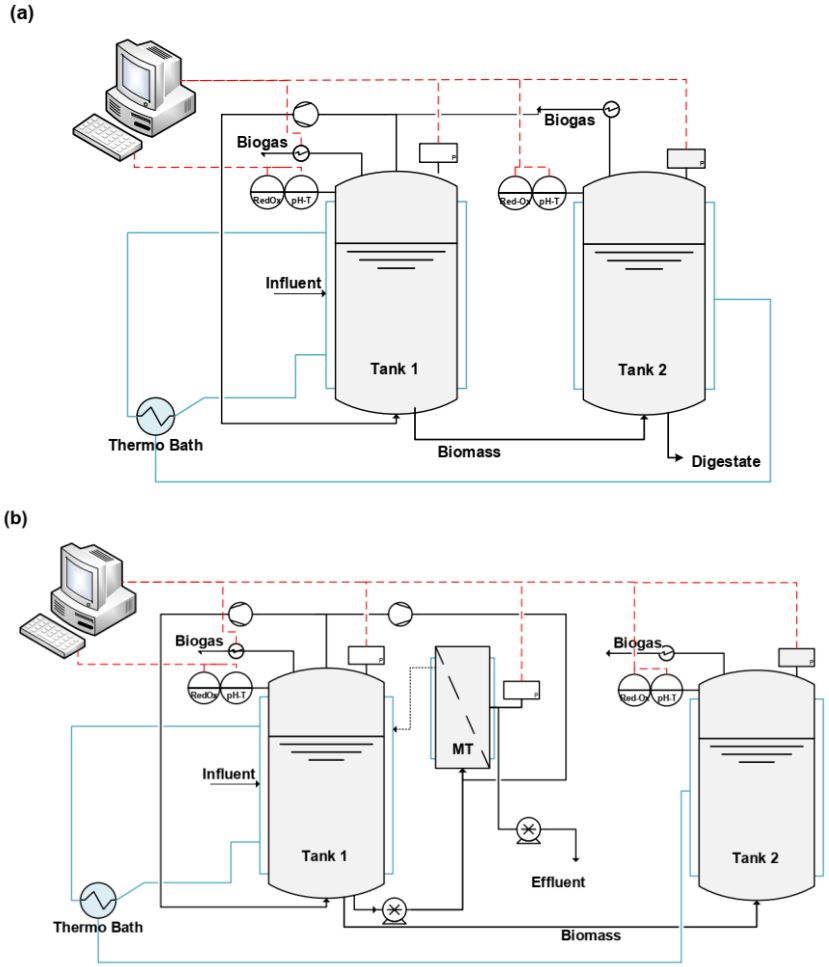

Figure 6.1. Anaerobic system layout: (a) CSTR+CSTR and (b) AnMBR+CSTR configuration. In figure b the system is composed of a main tank (Tank 1) and a coupled membrane tank (MT) (AnMBR) plus the reservoir (Tank 2, CSTR).

During period 1 (Figure 6.1a), the system consisted of two continuous stirred tank reactors (CSTR+CSTR). The influent was firstly degraded in the first CSTR (Tank 1) with an SRT ranging between 7 and 28 days. The

digestate extracted from Tank 1 to maintain this SRT was stored in the second CSTR (Tank 2). This second tank was not considered for process performance analysis, as explained before. After 56 days of operation an external ultrafiltration hollow-fiber module was coupled to Tank 1 (transforming the CSTR+CSTR configuration into an AnMBR+CSTR system) to evaluate high SRT influence over the microbial community without increasing the HRT. The new AnMBR+CSTR configuration of the anaerobic system is shown in Figure 6.1b. During Period 2 the SRT was increased and studied between 70-100 days while maintaining an HRT of 30 days (the rest of the operational conditions remained the same, as can be seen in Table 6.1). Finally, to evaluate the effect of a higher microalgae load over the microbial population, the HRT was decreased to 15 days in Period 3 to increase 2-fold the OLR of the system. The rest of the operational conditions and the AnMBR+CSTR configuration were maintained.

Table 6.1. Operational conditions of the rumen inoculated bioreactor.

	Period 1	**Period 2**	**Period 3**
Reactor configuration	CSTR+CSTR	AnMBR+CSTR	AnMBR+CSTR
OLR (g COD·L^{-1}·d^{-1})	0.2	0.2	0.4
HRT (d)	7-28	30	15
SRT (d)	7-28	70-100	100
Duration (d)	56	149	231
Biomass collection days (d)	0, 8, 42	92, 106, 155, 169, 190	339, 435

6.2.2. Microalgae source

A photobioreactor pilot plant located in Valencia (Spain) was used as a source of microalgae biomass. This plant is used as a tertiary treatment of sewage in "Carraixet WWTP" (Valencia, Spain) (Viruela *et al.*, 2017). This plant is fed with the anaerobic effluent of an anaerobic membrane bioreactor treating sewage, which is characterized by high nutrient concentration (Seco *et al.*, 2018). The mixed phototrophic culture is dominated by *Scenedesmus* spp. (99% relative abundance), according to microscopic observations of the phytoplankton. These microalgae grow spontaneously in the plant conditions.

As detailed in Giménez *et al.* (2017), the algae were concentrated up to 6093 g $TS \cdot L^{-1}$ after being harvested within a crossflow ultrafiltration hollow-fiber (CFUHF) membrane unit (Koch Romicon 2″, 0.03 μm pore size). The resulting biomass was stored at 4ºC for no longer than two weeks and daily fed to the system according to the established OLR (Table 6.1).

6.2.3. Performance analysis: physicochemical analysis and biogas production

The system performance was evaluated using digestate and effluent samples to determine the concentration of TS, VS, total suspended solids (TSS), total and soluble COD (T-COD and S-COD respectively), sulphate ($S\text{-}SO_4$) and nutrients (ammonium as NH_4-N and phosphate as PO_4-P), according to the standard methods (APHA, 2012). Additionally, carbonate alkalinity and volatile fatty acids were determined following the titration method of the South African Water Research Commission (Moosbrugger *et al.*, 1993).

Biogas was continuously measured using a μflow® gas flow meter (Bioprocess Control, Sweden). The biogas from Tank 1 and Tank 2 headspaces was sampled three times per week. The methane content of the biogas was measured using a gas chromatograph fitted with a flame ionization detector (GC-FID, Agilent Technologies, USA). The gas sample (0.25 mL) was taken using a gas-tight syringe through a sampling point located on the top of each tank. The GC-FID was equipped with a TRACER column (Teknokroma) of 15 m x 0.53 mm x 1 μm dimensions and 40ºC temperature. Helium was chosen as the carrier gas with a flow rate of 5 $mL \cdot min^{-1}$. The standards for methane quantification were prepared with high pure (99.99% purity) methane gas (Air Products Inc.).

6.2.4. Biomass collection and 16S rRNA gene sequencing

Digestate samples for microbial analysis were collected from the two main 14.0 L tanks conforming the system (Figures 6.1a, 6.1b) after 0, 8, 42, 92, 106, 155, 169, 190, 339 and 435 days of operation (Table 6.1). Digestate pellets obtained after 10 minutes centrifugation at 5000 x g were stored at -

20°C and later used for nucleic acid extraction within E.Z.N.A DNA Extraction Kit for Soil (Omega-Biotek, USA), according to the manufacturer's protocol. Nucleic acid from the reservoir at collection point 106 days was extracted in duplicate and included as a control of the nucleic acid isolation stage (sample 106-Rb). After fluorometric dsDNA quantification assay with Qubit 2.0 (Thermo Scientific, USA), Illumina amplicon libraries were generated using indexed primers that target the 16S rRNA gene (Takahashi *et al.,* 2014). The 2x300 bp run was performed within an Illumina MiSeq using v.3 chemistry (Illumina, USA) in FISABIO next-generation sequencing service (Valencia, Spain). Collected samples from Tank 1 (day 155) and reservoir Tank 2 (days 106 and 155) were used as a control of the sequencing stage. Nucleic acid from these three samples was sequenced in a different run, using the same conditions for library preparation, Illumina sequencing chemistry and machine. All sequences retrieved were deposited on the NCBI Sequence Reading Archive (SRA) database under bioproject number PRJNA434206 (accession numbers SAMN11567577-96).

6.2.5. Amplicon sequencing downstream analysis

A downstream high-quality sequencing data analysis based on the fastq-score of each read ($q \geq 30$ threshold) was applied to the sequences retrieved from the Illumina platform as previously described (Zamorano-López *et al.*, 2019c). The resulting operational taxonomic units ($OTU_{0.97}$) were generated in an open-reference clustering step at 3.0% dissimilarity. Taxonomic assignment was performed according to the 16S rRNA-based LTP 128 release of SILVA in QIIME. Phytoplankton related reads (Chloroplast and *Cyanobacteria*) were removed before downstream analysis since they are mainly related to the microalgae feedstock used in this study and might not be functional in an anaerobic system due to the absence of both oxygen and light. Besides, these reads are commonly associated to primer biases. As well, $OTU_{0.97}$ below 0.01% relative abundance percentages were excluded from analysis to reduce the background noise effect of rare reads.

The 16S rRNA gene analysis was performed over rarefied sequences to the minimum depth achieved (17,993-125,892 raw reads) to exclude the effect of differences in the sequencing depth per sample. The microbial community structure was evaluated first calculating the weighted unifrac distances between samples according to the observed species and later analyzing the distance matrix in a principal co-ordinate analysis (PCoA). The different community structures observed were statistically evaluated in an analysis of similarities test (ANOSIM). The link between operational parameters, digestate and feedstock physicochemical characteristics and microbial community dominant members (over 0.5% relative abundance) was performed through sparse partial least square analysis (sPLS). As a result, a relevance network and a pair-wise correlation heatmap were constructed using the retrieved sPLS regression model, showing the correlation between both biological and physicochemical data matrixes (González *et al.,* 2013).

6.3.Results and Discussion

6.3.1. Acclimatised biomass from a rumen inocula at high solids retention time improves raw Scenedesmus conversion into biogas

Figure 6.2 shows the relative abundance of the different phyla identified in the AnMBR along the complete experimental period. As can be seen in the figure, the potential for *Scenedesmus* biomass conversion into biogas was mainly attributed to *Bacteroidetes, Chloroflexi, Cloacimonetes, Euryarchaeota, Firmicutes, Proteobacteria, Spirochaetae* and *Thermotogae* phyla. Most of these groups were not only observed during the first stages of the rumen system, but also remained during the whole experience. The enhance and persistence during the studied period of these groups, especially *Thermotogae* and *Chloroflexi*, might have helped the system to achieve the 62% biodegradability values of raw *Scenedesmus* (Table 6.2).

Table 6.2. Performance mean and standard deviation values of the rumen inoculated system.

		Period 1	Period 2	Period 3
Biodegradability*	%	32±4	62±4	49±3
COD removal	%	36.1±8.8	70.1±10.7	57.2±1.4
Methane Yield	$mLCH_4 \cdot gCOD^{-1}$	110±24	214±15	177±11
Methane Yield	$mLCH_4 \cdot gVS^{-1}$	185±45	360±52	305±16

*Calculated based on by-product COD over total influent COD, as detailed in Giménez *et al.*, 2017. Further data available in supplementary Table 6.4.

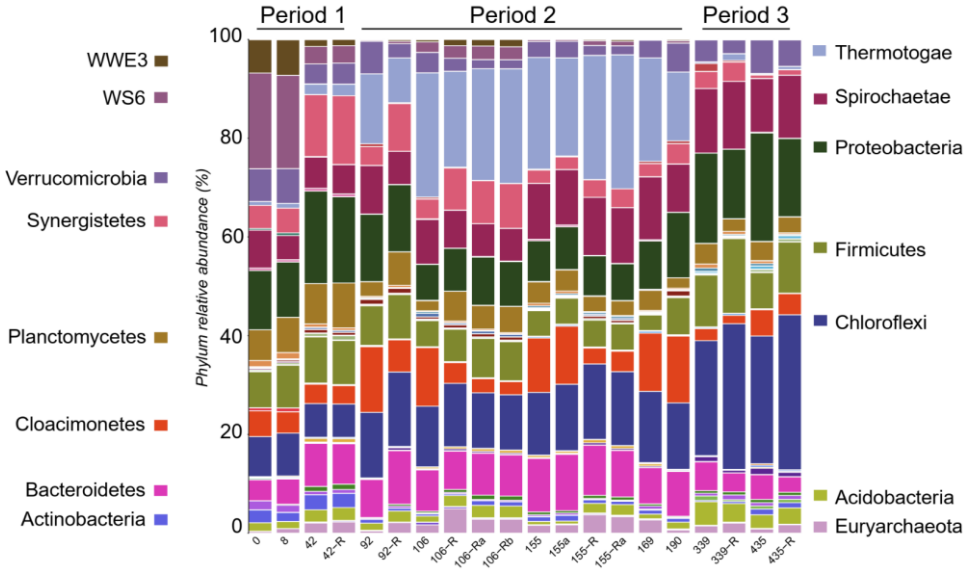

Figure 6.2. Relative abundances of the different phyla identified in the AnMBR. Samples collected from the biomass reservoir are indicated as "-R". Samples collected after 106 and 155 days were duplicated to be used as control between different Illumina runs (labels 106-Ra, 155a and 155-Ra). Reservoir sample collected after 106 days was extracted twice and included as a control of the nucleic acid isolation, library preparation and 16S rRNA sequencing (label 106-Rb).

The modification of the treatment scheme from a CSTR+CSTR system operated at low solids retention time (7-28 days) in Period 1 to an AnMBR+CSTR system with higher solids retention time (70-100 days) in Period 2 shifted the 16S community composition profiles. Microbial groups with slower growth rates but high hydrolytic potential like *Thermotogae*

were then enhanced and remained in the system while maintaining SRT at 100 days and an OLR of 0.2 $g \cdot L^{-1} \cdot d^{-1}$. Interestingly, the biodegradation potential increased in the system 2-fold as the SRT was increased through membrane operation from Period 1 to Period 2. Dominant phyla found during this period ranged as follows: 9.1-27.1% *Thermotoga*, 7.3-11.4% *Bacteroidetes*, 11.2-15.3% *Chloroflexi*, 2.7-13.5% *Cloacimonetes*, 3.1-9.0% *Firmicutes*, 7.3-13.6% *Proteobacteria* and 6.6-12.8% *Spirochaeta* (Table 6.3, supplementary). Besides, the higher detection of methanogens was observed after coupling the membrane tank to the system and increasing the SRT (Period 2). Under AnMBR+CSTR configuration the *Euryarchaeota* phylum (where the methanogens found here were classified) accounted for maximum relative abundance values of 5.0%. According to this result, high SRT (over 70 days) allows a good acclimation of the biomass boosting slow-growing microorganisms like potential hydrolyzers and methanogens in the system, establishing a more positive scenario for raw *Scenedesmus* conversion into biogas.

Natural hydrolytic consortia like the ruminal fluid can improve the hydrolysis efficiency of the first stages of anaerobic digestion, triggering the consequent stages and enhancing methane production (Barragán-Trinidad *et al.*, 2017). These authors demonstrated that ruminal fluid taken out from a cow enhanced a 29% the hydrolysis rate, resulting in a 193 mL $CH_4 \cdot gCOD^{-1}$ methane yield in a two-stage anaerobic digestion process. In the present work, the values obtained after rumen acclimation at high SRT accounted for 214 mL $CH_4 \cdot gCOD^{-1}$. This methane yield is very similar to the values reported by Mendez *et al.* (2014), who applied an enzymatic treatment stage to the *Scenedesmus* biomass prior to its anaerobic digestion. In the present work, 305 $mLCH_4 \cdot gVS^{-1}$ were produced from a robust microalga without pretreatments. This methane yield is higher than the ranging values between 127-258 $mLCH_4 \cdot gVS^{-1} \cdot L^{-1}$ summarized by Klassen *et al.* (2016) using untreated *Scenedesmus* biomass under mesophilic conditions. Only the study from Frigon *et al.* (2013) reached a higher value of 397 $mLCH_4 \cdot gVS^{-1}$. However, as pointed by the authors, previous freezing stage due to

microalgae transportation could have enhanced the methane yield in the experiment. Hence, it is worth highlighting that the use of the ruminal fluid inoculum in the AnMBR to convert raw *Scenedesmus* in biogas avoids the associated economic cost to the pretreatment stage of the biomass. This strategy should be therefore considered for industrial systems.

6.3.2. Rumen inoculum role during the early stages of anaerobic digestion

The resulting biomass retained and enhanced in the early stages of the rumen inoculated bioreactor was mainly composed of *Leptospiraceae* (*Spirochaeta* phylum), *Planctomycetaceae* and *Pirellula* (*Planctomycetes*), *Synergistaceae* (*Synergistes*), *Gelria* (*Firmicutes*) and other uncultured members from *Bacteroidetes*; besides WS6 and WWE3 (Figure 6.3). Little is known about WS6 and WWE3 phyla, recently proposed as *Candidate Dojkabacteria* and *Ca. Katanobacteria*, respectively. Their potential implication in hydrolytic pathways has been suggested using a metagenomic approach. Pandit *et al.* (2016) determined that both phyla contain encoding genes for degradation of chitin, xylose, cellobiose and hemicellulose. Some of these complex compounds are commonly found in *Scenedesmus* cell bodies (Baudelet *et al.*, 2017). In the present work, the relative abundance of WS6 and WWE3 groups were remarkable only between 0-8 days of operation (19.4% WS6 and 7.2% WWE3 maximum relative abundance values). However, both groups were washed out during the performance at higher SRT, which was increased from 28 days to 70-100 days. During Period 1, only a 32% biodegradability value was reached in the rumen inoculated system. This value is slightly higher than the 22-24% values reported by González-Fernández *et al.* (2015) under mesophilic conditions for raw *Scenedesmus.* However, this is a very low value that corresponds only to 110 mL $CH_4 \cdot gCOD^{-1}$ methane yield (see Period 1 in Table 6.2). Thus, *Scenedesmus* cell walls and organelles were poorly disrupted when operating between 7-28 days SRT and HRT. This was mainly attributed to the washed-out of the main hydrolytic potential groups like WS6 and WWE3.

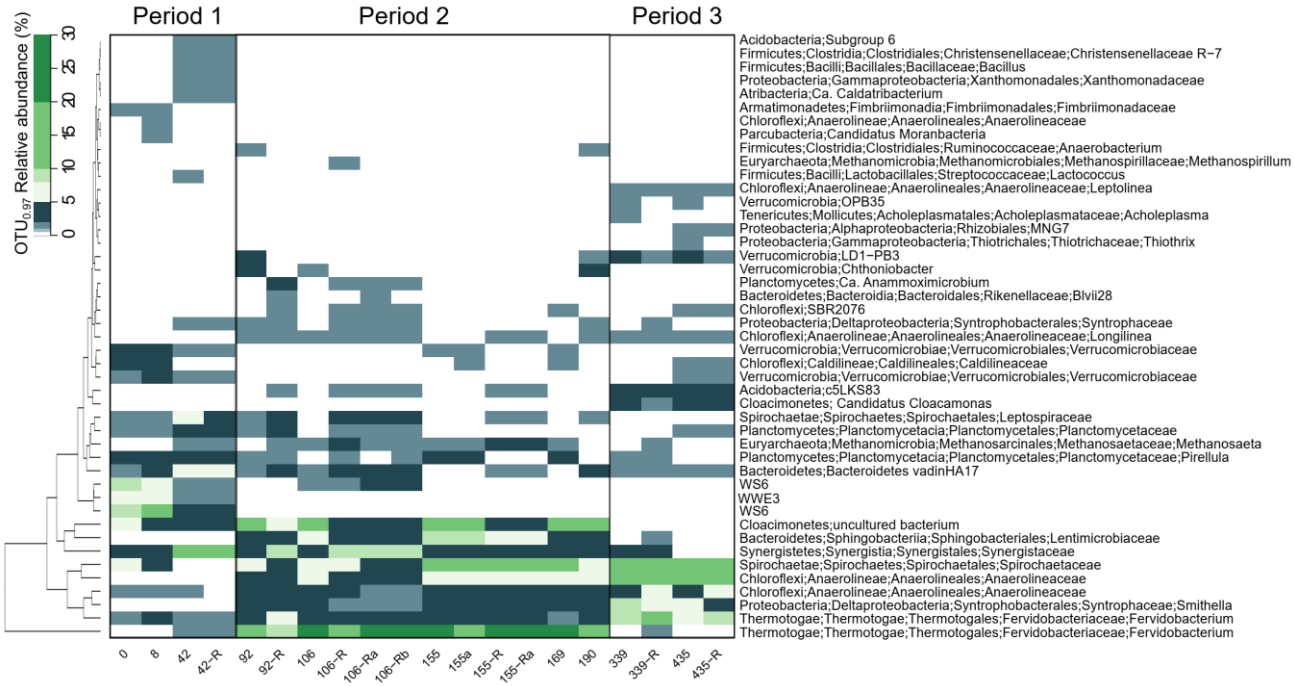

Figure 6.3. Relative abundance of the main $OTU_{0.97}$ identified during performance of the rumen-inoculated system. A blue palette has been used to differentiate minor groups (0.5-5.0% relative abundances) from dominant $OTU_{0.97}$ which are represented in greens (5.0-30.0%). Sample label indicates the collection day according to the continuous performance and samples taken from the reservoir are indicated as -R. Left-side cluster indicates similar patterns of relative abundances. On the right side appears the corresponding taxonomy from phyla to the minimum taxonomic level assigned to each $OTU_{0.97}$.

The anaerobic digester environment differs from the ruminant cavities. Instead, several groups such as *Bacteroidetes*, *Proteobacteria* and *Firmicutes* trend to be dominant (McIlroy *et al.*, 2017). Despite the presence in the system of interesting groups for microalgae cell wall disruption, not all of them were selected yet they were replaced by others. Moreover, the composition of *Scenedesmus* cells is unique and complex due to the presence of algaenan (Baudelet *et al.*, 2017; Carrillo-Reyes *et al.*, 2016) and might have had a substrate-specific selective effect over rumen dominant microorganisms. Hence, a long-term operation for biomass acclimation to the characteristics of *Scenedesmus* biomass was required to enhance the performance in terms of biodegradation and consequent energy recovery as biogas.

6.3.3. Key role of Fervidobacterium for Scenedesmus disruption at 39°C

After a first acclimation stage of the ruminal fluid in the reactor, microbial groups with potential affinity for *Scenedesmus* disruption were stabilized in the system. The coupling of the membrane tank allowed to increase the SRT up to 70 and 100 days (maintaining the HRT in 30 days). This operational change increased the biodegradability values observed from 32% to 62% in Period 2 (Table 6.2). A remarkable change in the population was attributed to the relative abundance of *Thermotoga* phylum, that peaked during Period 2 and reached relative abundance values up to 26.8% in the system. The remarkable *Thermotoga* presence was attributed to one single $OTU_{0.97}$ closely related to a *Fervidobacterium* strain isolated from a full-scale digester located in Arizona, USA (SILVA accession number FJ769489.1.1476). *Fervidobacterium* genus has been found in a mining study for detection of genes and microbial taxa involved in complex biopolymer degradation, like hemicelluloses (Pandit *et al.*, 2016). Also, this genus has been related to a primary fermenting lifestyle, releasing acetate, hydrogen and carbon dioxide end-products (Wushke *et al.*, 2018). However, further research focused on proteomic and metabolomic analysis would be needed to explore the catabolic implications of *Fervidobacterium* during *Scenedesmus* cells decomposition in the present work.

To the current knowledge of the authors of this manuscript, no other studies have reported before the role of *Fervidobacterium* in a similar biological process for microalgae conversion into biogas. This could be related to the temperature fixed in this study (39°C), which differs to other similar studies that are closer to 35°C or 55°C when evaluating mesophilic or thermophilic conditions, respectively (González-Fernández *et al.*, 2018; Klassen *et al.*, 2016). Nevertheless, the remarkable abundance of this group in the acclimatised rumen system suggests its potential role during raw microalgae anaerobic digestion.

6.3.4. Anaerolineaceae, Spirochaetaceae, Lentimicrobiaceae and Cloacimonetes members control raw Scenedesmus anaerobic digestion at high SRT

Together with *Fervidobacterium*, members of *Anaerolineaceae*, *Spirochaetaceae*, *Lentimicrobiaceae* and *Cloacimonetes* conformed a unique microbial community structure in the AnMBR+CSTR system operated at high SRT. *Anaerolineaceae* microorganisms have a fermentative metabolism and have been previously related to the degradation of microalgae biomass, including *Scenedesmus* in continuous anaerobic systems at mesophilic temperatures. Interestingly, *Anaerolineaceae* were also observed when degrading raw *Scenedesmus* with an acclimatised mesophilic sludge inoculum at SRT of 100 days reaching 40% relative abundance values (Greses *et al.*, 2017). Furthermore, Sanz *et al.* (2017) determined the dominance of this family (22.6-25.0%) in different CSTR treating a *Chlorella* biomass at SRT of 15 days . In the present study, an $OTU_{0.97}$ related to *Methanosaeta* was observed in the system ranging 0.4-3.5% relative abundances. These results suggest the relevance of methane producing pathways that are dependent to acetate-producing fermentative partners like *Anaerolineaceae* members. The fermentative metabolism of *Anaerolineaceae* was reported from genomic anotation, while their syntrophic interaction with methanogens like *Methanosaeta* was demonstrated through rRNA fluorescence-in situ hybridation by Mc. Ilroy *et al.* (2017). The authors observed that both *Methanosaeta* and

Anaerolineaceae members are filamentous and tend to agreggate in anaerobic environments. This association might be enhanced in AnMBR as a result of the biofouling development in the membrane tank through cycle combination of filtration and backwashing.

Besides saccharolytic members of *Chloroflexi*, other uncultured groups related to *Spirochaetaceae* and *Lentimicrobiaceae* were observed. A recent study, focused on bioaugmentation with rumen-related microorganisms for lignocellulose degradation, highlights the potential role of *Spirochaetaceae* uncultured members for volatile fatty acid production in anaerobic digesters (Deng *et al.*, 2018). Values ranging 0.8-12.4% of an $OTU_{0.97}$ related to this family were observed during Period 2 in this work. Bacteroidetes members were mainly attributed to the *Lentimicrobiaceae* member (up to 9.9% presence), that encompasses uncultured bacteria able to degrade complex polysaccharides such as starch at high-loaded waste streams (Sun *et al.*, 2016). Finally, another dominant group related to an uncultured *Cloacimonetes* was found between 1.7-13.5 % relative abundance values. Members belonging to this group are widely extended in anaerobic digestion systems, according to a recent study of 20 mesophilic full-scale bioreactors (Calusinska *et al.*, 2018a). Despite of the lack of further metabolic information, the evidences found in the present work suggest their important role for *Scenedesmus* degradation and their enhancement from rumen inoculum.

After inoculating the present anaerobic system with ruminal fluid, several microbial groups were retained and gradually enhanced as the SRT was being increased up to 100 days SRT, developing an efficient acclimatised biomass for raw *Scenedesmus* disruption. Gimenez *et al.* (2017) previously demonstrated the favorable effect of high solids retention over the biodegradability capacity of the system. Now, the microbial analysis here reported reveals the composition of the resulting AnMBR microbial community. The presence of microbial groups capable of perform the hydrolysis of complex polysaccharides as *Fervidobacterium*, *Anaerolineaceae*, *Lentimcirobiaceae*, *Spirochaetaceae* and *Cloacamonas*

also supports the favorable effect of high SRT achieved in the AnMBR for boosting biomethanization of microalgae. The configuration of the reactor should also be carefully considered, as biofouling of membrane systems promotes substantial changes in microbial communities and stimulate methanogenic-niche generation (Smith *et al.*, 2015).

Biofouling in AnMBR systems is still poorly understood from a microbial ecology perspective. However, the importance of direct interspecies electron transfer (Lovley, 2017) in these bioreactors should be considered when degrading microalgae. As pointed out by several authors, adhesion capacity of microorganisms to the plant fibers is crucial for their disruption (Yue *et al.*, 2013). Hence, biofouling in the AnMBR might have promoted aggregation between *Scenedesmus* cell-bodies and microbial groups with cellular attachment capacity like *Anaerolineaceae* (Xia *et al.*, 2016). In fact, this group was enhanced in the digester in Period 2 from 6.7% to 15.3% relative abundance. Finally, cellular adhesiveness might have facilitated the transference of metabolites between hydrolytic and primary fermenters to other groups involved in later stages of anaerobic digestion such as syntrophic-oxidizing bacteria and methanogens.

6.3.5. High solids retention time achieved in the AnMBR shaped the microbial community structure.

High SRT with a maximum of 100 days was achieved in the AnMBR in Periods 2 and 3. The effect of this important parameter over the rumen digester microbial community structure was evaluated through beta diversity ecological analysis (Figure 6.4).

The first two PCoA components explain the 78% of the variability between the rumen system samples analyzed. The system configuration significantly shaped the microbial community structure, as three different clusters were observed (ANOSIM statistic R 0.9762; $p<0.001$). The analysis of the biomass reservoir samples reveals the stability of the community structures observed in the three periods. As can be seen in the PCoA, samples taken from the reservoir show the same community structure

changes than those collected from the main tank among periods. Slight differences observed between these samples in Period 2 might be related to the higher retention times of this reservoir tank than the main tank since the membrane tank was not included.

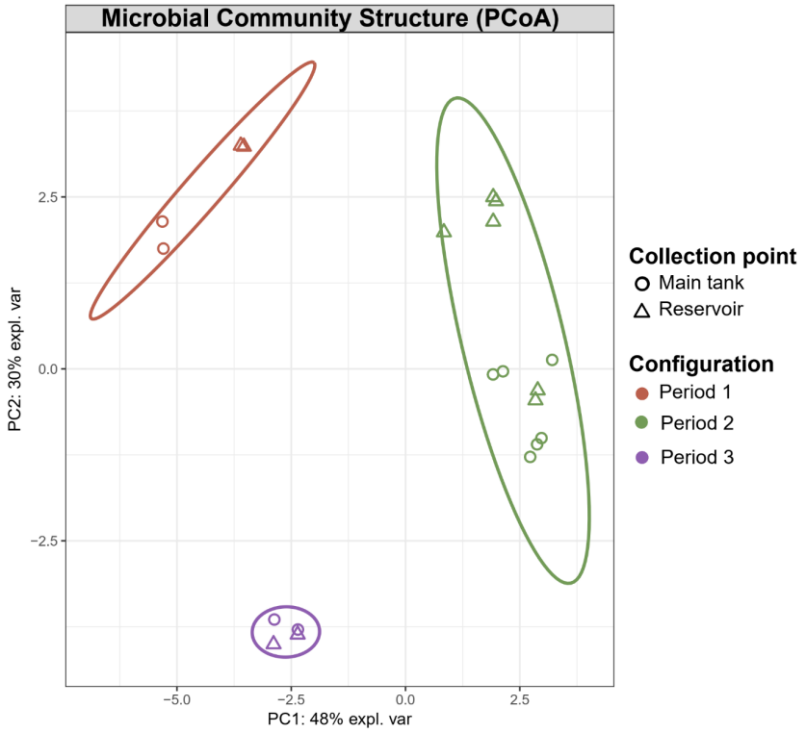

Figure 6.4. Principal Co-ordinate Analysis (PCoA) ordination plot of the weighted UniFrac distances observed between microbial community members of the rumen system. The first two components plotted explain 78% of the variability among samples. Collection points are differentiated using circles (Tank 1, main tank) and triangles (Tank 2, reservoir tank). Ellipses show 0.95 confidence areas estimated through a multivariate t-distribution of the data (ANOSIM statistic R: 0.9762; Significance: $p<0.001$).

Presumably, microbial population was shaped by the synergistic effects of biomass acclimation to microalgae composition and SRT over 70 days. Microorganisms selection when long-term degrading a specific substrate is thus an important parameter that shapes biogas producing microbial communities. However, other relevant parameters like the OLR have a secondary effect over these microbial structures as a different structure was

observed in Period 3 despite maintaining the SRT at 100 days in the AnMBR+CSTR configuration. The absence of the key microorganism *Fervidobacterium* and the increase of key *Anaerolineaceae* and *Spirochaetaceae* members are the responsible for this structural change. As reported by Muñoz-Sierra *et al.* (2018), the use of AnMBR to adapt anaerobic biomass to specific and complex compounds promotes strengthened microbial structures and end up in process optimization. In fact, these community structures are robust over-time. This can also be concluded in the present study, as no diversity differences have been found between the samples taken from the pseudo-steady periods studied.

6.3.6. Linking microbial community and operational parameters during Scenedesmus biomethanization

The sPLS analysis allowed the elucidation of a relevance network based on the performance data retrieved from the system during the studied periods and the $OTU_{0.97}$ relative quantification (Figures 6.5 and 6.6 in Supplementary Information). The sPLS regression model was constructed using the first two components extracted (38.0% and 31.0% of explained variance). Similarity between the samples distribution based on sPLS and PCoA analysis highlights the importance of the community structure for the better performance of the digester found during Period 2. A negative correlation between several groups and the COD removal variable reveals those members that were not selected for *Scenedesmus* conversion, *Ca. Dojkabacteria* and *Katanobacteria* among others. In contrast, a positive correlation is shown in the analysis between *Leptolinea* (phylum *Chloroflexi*, family *Anaerolineaceae*) and the methane yield determined during the experience. This parameter and the SRT have a close distance in the network analysis, showing the relationship between the favourable effect of high SRT and system performance in terms of methane production. Interestingly, a very high correlation was elucidated from the sPLS analysis between *Fervidobacterium*, a *Lentimicrobiaceae* member and HRT. Both $OTU_{0.97}$ were outcompeted and washout from the system when decreasing the HRT from 30 to 15 days and enhancing 2-fold the OLR. A progressive

increase of the OLR could have mitigated the effect of a feedstock overload over these groups. On the other hand, the network analysis shows a positive correlation between *Smithella*, *Gelria* and *Methanolinea* and the OLR. Although correlation does not necessarily indicate causation, these results suggest the potential role of these groups during the system response to a *Scenedesmus* feedstock overload.

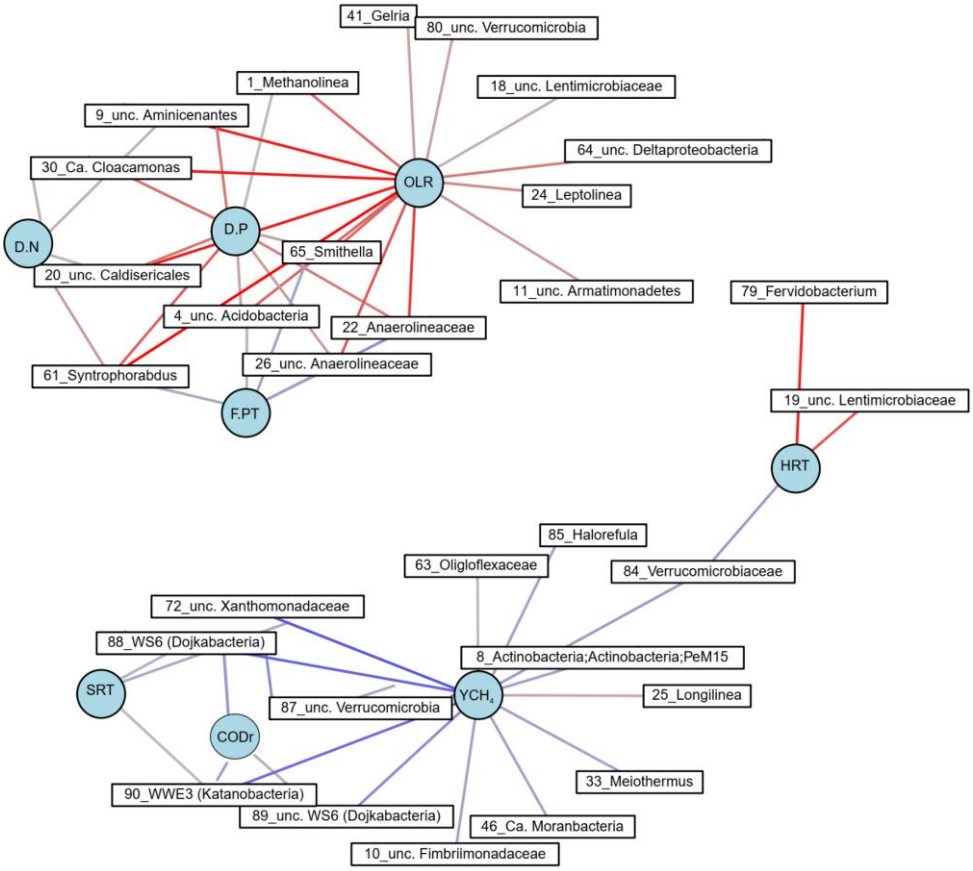

Figure 6.5. Relevance network from sPLS analysis. Threshold value is 0.65. Positive and negative correlation is shown through red and blue lines between nodes, respectively. The higher intensity of these colors, the higher correlation value. Circle-nodes are significant operational parameters like organic loading rate (OLR), hydraulic retention time (HRT), solids retention time (SRT); or quantitative measurements of Total Nitrogen and Phosphorous from digestate (D.N and D.T) and feedstock (F.PT), methane yield (YCH_4), and COD removal (CODr). Boxes contain the significant $OTU_{0.97}$ selected by the sPLS regression model.

a. Dominance of acetoclastic methanogens during raw *Scenedesmus* biomethanization

After *Scenedesmus* hydrolytic disruption, released components are converted into methanogenic substrates such as hydrogen, carbon dioxide and fatty acids (mainly acetate). A fast dominance of the *Methanosarcinales* group was detected in the system, reaching relative abundance values up to 5.0% (E-supplementary data). Acetoclastic capacity for methane production is specifically attributed to different members of this group such as *Methanosarcina* and *Methanosaeta* (Schmidt *et al.*, 2016). *Methanosaeta*, the dominant methanogen observed in this work, has been also identified as the main methane producer from acetate in similar studies degrading microalgae under mesophilic conditions (Greses *et al.*, 2017; Klassen *et al.*, 2016; Zamalloa *et al.*, 2012b). In this work, the biodegradability experimented a 2-fold increase from Period 1 to Period 2, suggesting that acetate released from the fermented *Scenedesmus* hydrolyzed compounds was quickly cleaved by *Methanosaeta* into methane and carbon dioxide. The upward trend of this $OTU_{0.97}$ seems to be positively correlated with the biomethanization enhance observed (E-supplementary data), until reaching its maximum value of 214 mL $CH_4 \cdot gCOD^{-1}$. Hence, the importance of the acetoclastic pathways for methane production can be suggested from the 16S rRNA gene sequencing findings in this work. Finally, this is in accordance with the findings from Venkiteshwaran *et al.* (2015), that reported most of the methane produced in high solids retention systems (such as the municipal full digesters) comes from acetate.

a. Syntrophic-microorganisms response against a feedstock overload

Syntrophic acetogens play an important role during anaerobic digestion as they can convert intermediate products such as butyrate, propionate, lactate and ethanol in methanogenic substrates i.e. acetate, hydrogen, carbon dioxide and methyl compounds (Leng *et al.*, 2018). In the present work, the acetoclastic pathway was the main methanogenic reaction suggested according to the dominance of *Methanosaeta*. However, after increasing the

OLR during Period 3 lower values of this methanogen were observed (from 2.0% to values below 0.5%).

The methane yield obtained during Period 3 was lower than in Period 2 (177 vs 214 mLCH4·gCOD^{-1} respectively). The lack of the potential acetate-producing bacteria found in this work, *Fervidobacterium*, resulted in a more complex microbial network for methane production. In this period up to 9.5% *Smithella* (δ-*Proteobacteria*) and 13.2% *Gelria* (*Firmicutes*) syntrophic bacteria were observed in the system. Both $OTU_{0.97}$ had been observed during the whole experience, at relative abundance values below 5.0%. However, they showed up a fast response against the higher load of *Scenedesmus* fed to the AnMBR in Period 3 (OLR 0.4 gCOD·L^{-1}·d^{-1}).

Smithella is involved in the conversion of butyrate and propionate into acetate (Narihiro *et al.*, 2018). *Methanosaeta* could probably remain in the system as a result of the *Smithella* role in fatty acids transformation into acetate. The role of *Gelria* in anaerobic environments is less understood compared to *Smithella*. Up to date, no isotope-probing confirmation has been found that reveals its suggested metabolic implication. However, previous metaproteomic analysis proposed its role as a syntrophic hydrogen-producing bacteria during cellulose biomethanization (Lu *et al.*, 2014). Moreover, a recent transcriptomic study of municipal co-digesters also hypothesized its implication in syntrophic acetate oxidation of fatty acids. Although the biodegradability obtained in Period 3 did not reach the higher values previously found, the viability of continuous conversion of raw microalgae into biogas was still observed, accounting for 49% raw microalgae biodegradation.

Conclusions

High anaerobic biodegradability of raw *Scenedesmus* (62%) was reached using an acclimatised rumen inoculum. The importance of *Fervidobacterium* for microalgae disruption besides the release of intermediate products by *Anaerolineaceae*, *Spirochaetaceae*, *Lentimicrobiaceae* and *Cloacimonetes* was here highlighted. Acetoclastic

Methanosaeta and syntrophic groups thrived in the system allowing a good flux of acetate conversion into methane (305 $mLCH_4 \cdot gVS^{-1}$). The stabilization of the microbial structure and its hydrolytic potential supports the use of membrane technology in anaerobic systems to overcome operational limitations and benefit from the favorable effect of high solids retention time during anaerobic digestion of complex substrates.

Appendix A. Supplementary data

E-supplementary data of this work can be found in online version of the paper

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Table 6.3. Relative abundances of the dominant phyla and the relevant OTU0.97 found during continuous AnMBR performance.

Phylum	**Period 1**				**Period 2**												**Period 3**			
Taxonomic assignment	*0*	*8*	*42*	*42-R*	*92*	*92-R*	*106*	*106-R*	*106-Ra*	*106-Rb*	*155*	*155a*	*155-R*	*155-Ra*	*169*	*190*	*339*	*339-R*	*435*	*435-R*
Acidobacteria	**1.7**	**1.4**	**2.5**	**2.6**	**1.4**	**2.3**	**1.2**	**2.2**	**2.5**	**2.4**	**0.7**	**0.6**	**2.0**	**2.1**	**0.9**	**1.4**	**4.8**	**3.8**	**2.8**	**3.3**
unc. bacterium	-	-	-	-	-	1.5	-	1.7	2.0	1.8	-	-	1.7	1.9	-	-	4.6	3.7	2.4	3.2
unc. subgroup 6	-	-	1.4	1.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Actinobacteria	**2.6**	**1.8**	**3.0**	**2.9**	**0.8**	**0.6**	**0.4**	**0.3**	**0.9**	**0.9**	**0.9**	**1.1**	**0.7**	**0.7**	**0.8**	**0.8**	**0.3**	**0.4**	**1.2**	**1.0**
Bacteroidetes	**4.2**	**5.1**	**8.7**	**8.2**	**7.7**	**10.8**	**8.4**	**7.8**	**8.5**	**8.3**	**10.8**	**11.4**	**10.2**	**9.4**	**7.3**	**9.1**	**5.8**	**3.8**	**5.0**	**3.1**
unc.	1.3	2.1	6.7	6.4	1.8	4.5	1.3	2.5	3.3	3.4	-	-	1.6	1.5	-	2.3	1.2	1.0	1.9	1.4
unc. Rikenellaceae Blvii28	-	-	-	-	-	1.8	-	-	1.0	-	-	-	-	-	-	-	-	-	-	-
unc. Lentimicrobiaceae	-	-	-	-	3.8	3.8	5.1	3.7	3.6	3.4	8.7	9.0	7.8	7.1	4.7	4.6	-	1.0	-	-
Chloroflexi	**8.1**	**8.7**	**6.8**	**6.7**	**13.3**	**15.1**	**12.3**	**12.8**	**11.2**	**11.2**	**12.7**	**13.5**	**15.3**	**15.0**	**14.4**	**13.5**	**23.3**	**29.5**	**25.9**	**31.4**
unc. Anaerolineaceae	-	-	-	-	5.0	3.9	5.2	3.9	3.2	3.5	6.8	7.0	7.4	7.1	7.1	5.1	13.5	17.9	14.9	18.2
unc. Anaerolineaceae UCG-	-	1.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Leptolinea	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.8	1.6	1.2	1.3
Longilinea	-	-	-	-	1.2	1.8	1.2	1.8	1.3	1.4	-	-	1.3	1.2	-	1.3	1.1	1.5	1.2	1.5
unc. Anaerolineaceae	1.3	1.4	1.0	-	2.7	4.3	2.4	2.4	2.0	1.9	2.5	2.7	3.0	3.1	2.7	3.2	4.8	5.9	5.0	6.1
unc. Caldilineaceae	3.4	2.4	-	-	-	-	-	-	-	-	-	1.1	-	-	1.4	-	-	-	1.4	1.8
unc. Chloroflexi SBR2076	-	-	-	-	-	1.0	-	1.3	1.3	1.2	-	-	-	-	1.3	-	-	-	1.4	1.4
Cloacimonetes	**5.2**	**4.3**	**3.9**	**3.7**	**13.3**	**6.5**	**11.9**	**4.2**	**2.9**	**2.7**	**11.0**	**11.8**	**3.2**	**4.1**	**11.8**	**13.5**	**2.4**	**1.7**	**5.3**	**4.2**
Ca. Cloacamonas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.1	1.5	4.4	3.6
unc. Cloacimonetes	5.2	4.3	3.9	3.7	13.3	6.5	11.8	4.2	2.9	2.7	11.0	11.7	3.2	4.1	11.8	13.5	-	-	-	-
Euryarchaeota	**0.4**	**0.9**	**2.0**	**2.3**	**0.5**	**2.1**	**1.7**	**5.0**	**2.8**	**2.8**	**1.6**	**1.7**	**3.7**	**3.4**	**2.7**	**0.7**	**1.5**	**2.0**	**0.9**	**1.7**
Methanosaeta	-	-	1.4	1.6	-	1.5	1.5	3.5	1.9	1.9	1.5	1.6	2.5	2.3	2.0	-	-	1.1	-	-
Firmicutes	**7.3**	**8.6**	**9.3**	**8.9**	**8.0**	**9.0**	**5.3**	**6.6**	**7.9**	**7.9**	**5.2**	**5.3**	**5.5**	**5.3**	**3.1**	**7.5**	**10.5**	**15.1**	**7.3**	**10.4**
Gelria	1.9	3.5	2.0	1.9	4.7	6.7	3.6	4.8	4.7	4.8	3.7	3.7	4.3	4.3	1.3	4.1	8.4	13.2	5.6	9.1

(Continues in next page)

Phylum	**Period 1**				**Period 2**												**Period 3**			
Taxonomic assignment	*0*	*8*	*42*	*42-R*	*92*	*92-R*	*106*	*106-R*	*106-Ra*	*106-Rb*	*155*	*155a*	*155-R*	*155-Ra*	*169*	*190*	*339*	*339-R*	*435*	*435-R*
Planctomycetes	**6.2**	**7.1**	**8.2**	**9.1**	**3.0**	**6.8**	**2.0**	**6.0**	**4.8**	**5.3**	**4.3**	**4.3**	**3.1**	**2.9**	**4.1**	**2.1**	**4.1**	**2.5**	**3.8**	**3.2**
Ca. Anammoximicrobium	-	-	-	-	-	2.2	-	1.7	1.5	1.6	-	-	-	-	-	-	-	-	-	-
Pirellula	3.3	4.4	2.5	3.2	1.1	1.4	-	1.3	-	1.1	3.3	3.1	-	-	2.7	-	1.9	1.1	-	-
unc. Planctomycetaceae	1.4	1.2	2.8	3.0	1.0	2.2	-	1.9	1.6	1.6	-	-	-	-	-	-	-	-	1.6	1.3
Proteobacteria	**12.0**	**11.2**	**18.7**	**17.4**	**13.6**	**13.6**	**7.3**	**8.7**	**9.7**	**9.0**	**8.2**	**8.7**	**8.1**	**7.4**	**9.8**	**13.2**	**18.3**	**14.1**	**21.9**	**15.8**
Smithella	-	-	-	-	4.4	2.2	2.7	1.8	1.7	1.4	2.8	2.9	2.7	2.3	3.5	4.5	9.5	6.3	5.5	3.8
unc. Syntrophaceae	-	-	1.4	1.2	1.2	1.6	-	1.3	1.1	1.1	-	-	-	-	-	1.1	-	1.0	-	-
Spirochaetae	**7.7**	**4.9**	**6.3**	**5.9**	**9.8**	**6.7**	**9.1**	**7.7**	**6.6**	**6.6**	**11.3**	**11.2**	**11.8**	**11.3**	**12.8**	**9.7**	**13.0**	**13.7**	**10.8**	**12.8**
unc. Leptospiraceae	1.4	1.5	5.2	4.9	1.6	2.4	-	2.4	2.3	2.6	-	-	1.2	1.2	-	1.5	-	-	-	-
unc. Spirochaetaceae	6.2	3.3	-	-	7.6	4.0	7.8	5.1	4.2	3.9	10.6	10.5	10.5	10.1	12.4	7.6	12.9	13.5	10.8	12.6
Synergistetes	**4.7**	**5.0**	**12.6**	**13.9**	**3.8**	**9.7**	**4.0**	**8.5**	**8.7**	**9.0**	**2.7**	**2.6**	**3.6**	**3.8**	**2.6**	**4.1**	**3.4**	**3.9**	**0.7**	**1.1**
unc. Synergistaceae	4.2	4.7	11.8	13.3	3.7	9.2	3.8	8.2	8.4	8.7	2.6	2.5	3.4	3.6	2.6	3.9	2.8	3.6	-	-
Thermotogae	**0.7**	**0.9**	**2.1**	**2.3**	**14.2**	**9.1**	**25.2**	**19.4**	**22.6**	**23.2**	**22.6**	**19.9**	**25.2**	**27.1**	**21.0**	**13.9**	**0.3**	**1.3**	**0.2**	**0.6**
Fervidobacterium	-	-	1.8	2.0	13.8	8.4	24.9	18.7	22.1	22.6	22.5	19.8	24.7	26.8	20.9	13.6	-	1.0	-	-
Verrucomicrobia	**6.6**	**7.0**	**4.0**	**4.3**	**6.6**	**2.9**	**4.1**	**2.6**	**1.8**	**1.8**	**3.2**	**3.3**	**2.0**	**1.8**	**3.6**	**5.9**	**4.4**	**2.8**	**6.8**	**5.4**
unc. LD1-PB3	-	-	-	-	2.5	-	-	-	-	-	-	-	-	-	-	2.0	2.0	1.8	2.0	1.7
unc. OPB35 soil group	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.4	-	1.4	-
unc. Verrucomicrobiaceae	1.8	2.2	1.7	1.9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.4	1.4
unc. Verrucomicrobiaceae	2.7	2.8	1.1	1.1	-	-	-	-	-	-	1.4	1.5	-	-	1.0	-	-	-	-	-
WS6	**19.4**	**18.9**	**3.5**	**3.5**	**0.2**	**0.5**	**2.1**	**2.5**	**2.8**	**2.6**	**0.4**	**0.3**	**0.9**	**0.9**	**0.1**	**0.5**	**0.1**	**0.0**	**0.1**	**0.0**
unc. WS6	9.8	11.1	2.2	2.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
unc. WS6	9.5	7.7	1.4	1.4	-	-	1.8	1.9	2.1	2.0	-	-	-	-	-	-	-	-	-	-
WWE3	**6.8**	**7.2**	**1.4**	**1.2**	**0.1**	**0.3**	**0.5**	**1.2**	**1.3**	**1.5**	**0.0**	**0.0**	**0.2**	**0.3**	**0.0**	**0.2**	**-**	**-**	**-**	**-**
unc. WWE3	6.7	7.1	1.3	1.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 6.4. Mean values and standard deviation of the main parameters monitored during AnMBR performance.

		Period 1	**Period 2**	**Period 3**
Biodegradability*	%	32±4	62±4	49±3
CODremoval	%	36.1±8.8	70.1±10.7	57.2±1.4
pH		6.8±0.2	6.6±0.3	6.8±0.1
Total Nitrogen (digestate)	$mgN \cdot L^{-1}$	428.8±60.6	436.8±57.2	592.5±108.7
Total Phosphorous (digestate)	$mgP \cdot L^{-1}$	12.7±1.7	12.8±2.1	19.5±0.6
Total Nitrogen (feedstock)	$mgN \cdot L^{-1}$	410.8±12.5	318.3±59.6	367.5±8.7
Total Phosphorous (feedstock)	$mgP \cdot L^{-1}$	70.9±13.2	61.2±10.0	29.2±18.8

*Calculated based on by-product COD over total influent COD, as detailed in Giménez *et al.*, 2017

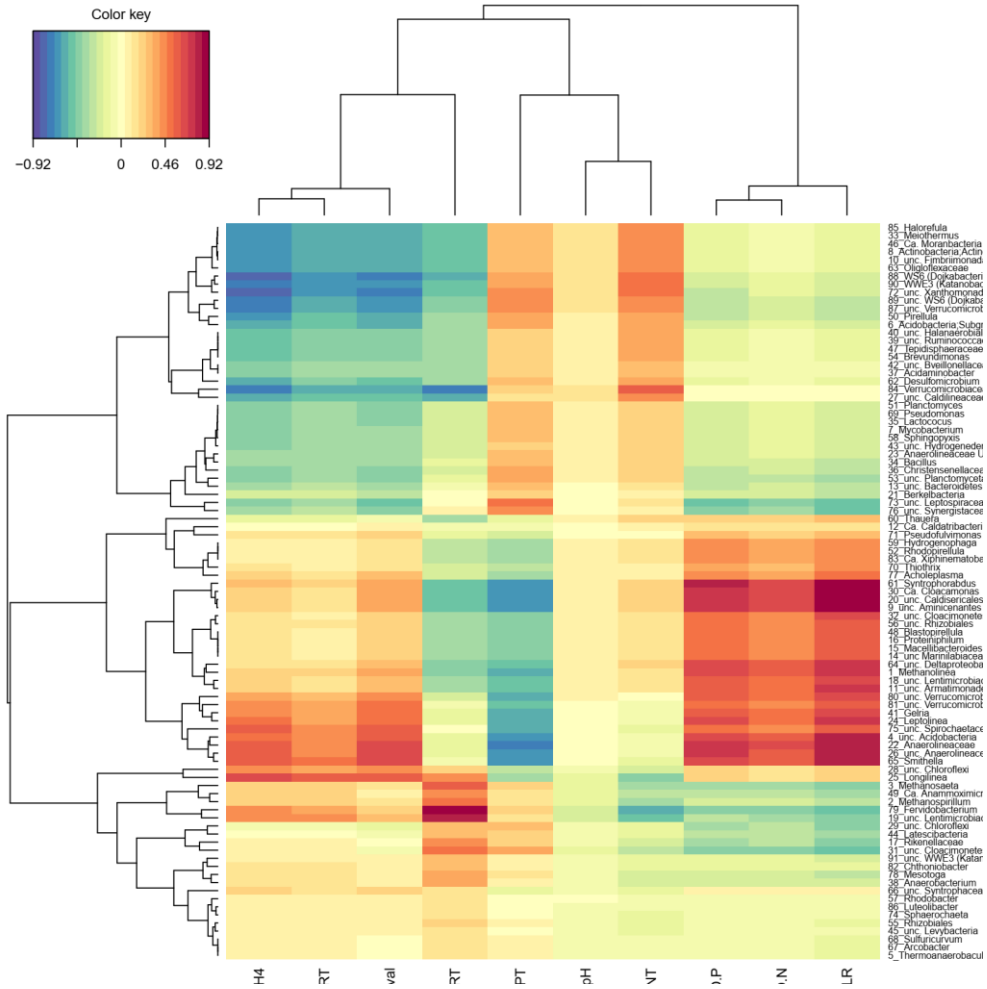

Figure 6.6. Pairwise correlation heatmap between the operational and performance data from the AnMBR (x axis) and the $OTU_{0.97}$ identified over 0.5% relative abundance values (y axis). Numeric labels refer to the sorted $OTU_{0.97}$ selected for sPLS analysis. The minimum taxonomic level assigned to each $OTU_{0.97}$ is shown in the right-side y axis. Labels of the x axis are: methane yield (YCH4), solids retention time (SRT), COD removal, hydraulic retention time (HRT), total phosphorous (F.PT) and nitrogen (F.NT) from feedstock, total nitrogen (D.N) and phosphorous (D.P) from digestate and organic loading rate (OLR). The cluster on the top of the heatmap associates the operational and physicochemical parameters according to their covariance. The left-side vertical cluster groups $OTU_{0.97}$ with similar trends in their relative abundance values.

7. Unveiling microbial structures during raw microalgae digestion and co-digestion with primary sludge to produce biogas using semi-continuous AnMBR systems

7. Unveiling microbial structures during raw microalgae digestion and co-digestion with primary sludge to produce biogas using semi-continuous AnMBR systems

Abstract

Methane production from microalgae can be enhanced through anaerobic co-digestion with carbon-rich substrates and thus mitigate the inhibition risk associated with its low C:N ratio. Acclimated microbial communities for microalgae disruption can be used as a source of natural enzymes in bioenergy production. However, co-substrates with a certain microbial diversity such as primary sludge might shift the microbial structure. Substrates were generated in a Water Resource Recovery Facility (WRRF) and combined as follows: *Scenedesmus* or *Chlorella* digestion and microalgae co-digestion with primary sludge. The study was performed using two lab-scale Anaerobic Membrane Bioreactors (AnMBR). During three years, different feedstocks scenarios for methane production were evaluated with a special focus on the microbial diversity of the AnMBR. 57% of the population was shared between the different feedstock scenarios, revealing the importance of *Anaerolineaceae* members besides *Smithella* and *Methanosaeta* genera. The addition of primary sludge enhanced the microbial diversity of the system during both *Chlorella* and *Scenedesmus* co-digestion and promoted different microbial structures. Aceticlastic methanogen *Methanosaeta* was dominant in all the feedstock scenarios. A more remarkable role of syntrophic fatty acid degraders (*Smithella*, *Syntrophobacteraceae*) was observed during co-digestion when only microalgae were digested. However, no significant changes were observed in the microbial composition during anaerobic microalgae digestion when feeding only *Chlorella* or *Scenedesmus*. This is the first work revealing the composition of complex communities for semi-continuous bioenergy production from WRRF streams. The stability and maintenance of a microbial core over-time in semi-continuous AnMBRs is here shown supporting their future application in full-scale systems for raw microalgae digestion or co-digestion.

Keywords

16S rRNA gene; anaerobic digestion; AnMBR; biogas; co-digestion; microalgae

Publication

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7.1.Introduction

The search for new sources of energy to reduce the CO_2 emissions of fossil fuels and mitigate this worldwide energy-dependence are among the principal motivations for moving forward more sustainable technologies and lifestyles. During the last decades, biofuel implementation has attracted the interest of the scientific community (Correa *et al.,* 2019). As a forward step, the concept of water resource recovery facilities (WRRF) has emerged for energy, nutrients, biosolids and reclaimed water recovery from sewage (Colzi Lopes *et al.,* 2018). Related to this concept, a promising water-energy nexus is the anaerobic treatment of sewage and the valorization of the resulting effluent for microalgae biomass generation (González-González *et al.,* 2018). This is a convenient loop, as microalgae can be harvested and later turned into biogas (González-Fernández *et al.,* 2015) in the previous anaerobic treatment stage (Xie *et al.,* 2018) or as a side-stream in future WRRF (Seco *et al.,* 2018a).

The biochemical composition of microalgae makes them suitable for bioenergy production through anaerobic digestion processes (Klassen *et al.,* 2016). However, pretreatment used to improve their biodegradability is expensive making the methane production from microalgae unfeasible (Carrillo-Reyes *et al.,* 2016). Therefore, feasible bioenergy generation from microalgae in future WRRFs needs biological strategies for microalgae cell disruption and degradation of the hydrolyzed components. Raw conversion of microalgae into biogas is possible when applying high solids retention times (SRT) in continuous bioreactors under mesophilic and thermophilic conditions (Greses *et al.,* 2018; Klassen *et al.,* 2016). As early reported by Zamalloa *et al.* (2012), the Anaerobic Membrane Bioreactor (AnMBR) allows to increase biomass retention whilst maintains low hydraulic retention times (HRT), making possible the continuous anaerobic digestion of microalgae.

As early remarked by Rivière *et al.* (2009), the definition of microbial cores in engineering systems can provide valuable information during operational parameter optimization processes. Zamalloa *et al.* (2012) was

the first work relying on microbial groups of microalgae anaerobic digestion through 16S rRNA gene fingerprinting. More recently, saccharolytic hydrolyzers and fermenters, as well as proteolytic bacteria from Bacteroidetes and Firmicutes phyla have been identified during *Chlamydomonas reinhardtii* anaerobic digestion (Klassen *et al.,* 2017). However, differences in the microalgae species can lead to different microalgae-degrading communities as their composition varies among their phylogeny (Baudelet *et al.,* 2017). Moreover, common microalgae that grow over sewage or anaerobic effluents have more resistant cell walls and can, therefore, require higher microbiological hydrolytic potentials. In this context, the acclimation of anaerobic sludge is a necessary step before the continuous conversion of microalgae harvested from sewage-related streams into biogas in WRRFs (González-Fernández *et al.,* 2018). The effect of the type of microalgae over the acclimated microbial community structures has not been thoroughly explored yet in the literature as most of the studies are focused on a single microalga.

The longer the SRT, the more favorable environment for slow-growing microorganisms that might be able to disrupt the microalgae cell walls (Greses *et al.,* 2017). However, more efficient biomethanization of microalgae could be obtained with more balanced C:N ratios through the addition of a co-substrate with high carbon content. The protein content of microalgae has an important drawback as the degradation of these compounds results in the release of nitrogen forms that can accumulate in anaerobic systems as free ammonia. Methanogens are sensitive to free ammonia and therefore, strategies to mitigate this inhibition risk are needed to enhance continuous energy production. According to Sialve *et al.* (2009), mass ratios between 20 and 35 have a positive effect over methane yield as well as over microalgae anaerobic digestion and mitigate the inhibition risk.

The favorable effect of co-digestion for microalgae anaerobic digestion was recently reported by Solé-Bundó *et al.* (2019). The authors achieved a 65% improved biomethanization when combining primary sludge and *Chlorella* biomass streams from a wastewater treatment plant. Also, the

degradation of *Scenedesmus* with pig manure resulted in a 50% increase in the methane yield (Astals *et al.*, 2015). Unfortunately, these studies did not evaluate the effect of SRT over microalgae co-digestion, despite the importance of this parameter to achieve high microalgae disruption rates (Greses *et al.*, 2018). Also, although several studies have explored different microalgae co-digestion scenarios (Herrmann *et al.*, 2016; Mahdy *et al.*, 2014b; Solé-Bundó *et al.*, 2018), none of them have been performed in a semi-continuous system operated under high SRT. Solé-Bundó *et al.* (2019) reported a 330 mL $CH_4 \cdot gVS^{-1}$ production from *Chlorella* and primary sludge in continuously stirred tank reactors (CSTR) but they applied a low SRT of 20 days and a protease treatment to the microalgae biomass. Furthermore, the microbiological aspects were not explored in the abovementioned systems and hence, there is a lack of knowledge on the different groups involved in microalgae co-digestion compared to single digestion. Only Li *et al.* (2017a, 2017b) reported the dominance of *Bacteroidetes*, *Proteobacteria*, *Firmicutes* and *Spirochaetae* during the co-digestion of *Chlorella* and chicken manure. However, this study applied a pre-treatment of the microalgae. As reported by Córdova *et al.* (2018), microalgae pre-treatment leads to important changes in microbial patterns, functionality, strategies, and interactions during microalgae anaerobic digestion. According to these authors, delta and gamma *Proteobacteria* were dominant for untreated *Chlorella* biomass digestion, but *Clostridia* was the most important group after applying an alkali-treatment to the same algal biomass. On the other hand, some of the co-substrates that can be added during microalgae digestion (*e.g.* primary and secondary sludge or manure) commonly have an inner microbial diversity that can disturb the microbial core developed during microalgae degradation. These aspects need to be evaluated in continuous systems to advance towards the design of management tools based on microbial community composition, like specific biomarker monitoring, in bioenergy production systems.

Several combinations of reactor configuration, temperature, SRT, HRT and feedstock composition have not been yet evaluated in the literature. In

our study, we use microalgae and primary sludge taken from a WRRF plant (Seco *et al.,* 2018) combining both anaerobic and microalgae technologies for sewage treatment. Although microalgae digestion has been thoroughly reported with reliance on the microbial populations (Córdova *et al.,* 2018; Klassen *et al.,* 2017; Sanz *et al.,* 2017), the microbial core for raw microalgae and primary sludge co-digestion has not been revealed in the literature. Furthermore, most of the studies including microbial characterization of systems for biogas production have been performed using traditional anaerobic digester configurations. On the contrary, the present work explores and characterizes the microbial communities of two semi-continuous AnMBRs converting raw microalgae into biogas. Hence, this study reveals important information about the stability over time of microbial populations acclimated to microalgae digestion and evaluates the effect over the microbial core behind this process when adding an extra carbon-source (such as primary sludge from the same WRRF) to balance the C:N ratio and mitigate the free ammonia inhibition risk. It should be highlighted that this is the first study reporting information obtained using the same acclimated biomass to degrade in a semi-continuous process two common microalgae grown on sewage streams such as *Chlorella* and *Scenedesmus* without any pretreatment.

7.2.Materials and Methods

7.2.1. Bioreactor operational conditions

Two different lab-scale mesophilic AnMBRs were operated to produce biogas from microalgae under the operational conditions summarized in Table 7.1. Both reactors were operated under mesophilic conditions (35°C). The first AnMBR (digester, Figure 7.6a) had a 12.4 L volume, 9.9 L working volume, considering the tank and the external hollow-fiber ultrafiltration membrane tank (0.42 m^2 surface, 0.05 μm pore size, PUR-ON® Koch Membrane Systems). The second AnMBR (co-digester, Figure 7.6b) had a 14 L volume (9 L working volume) and was equipped with an identical external membrane tank to the first AnMBR. A reservoir tank was coupled to the co-digester AnMBR and used for microbial analysis purposes as

detailed later. The digester was inoculated with mesophilic sludge from a full-scale digester located in the municipal WTTP Carraixet (València, Spain). The co-digester was inoculated with the stored biomass from the digester, available in the reservoir.

Table 7.1. Operational conditions of the two bioreactors used in this study.

		AnMBR digester		**AnMBR co-digester**	
Feedstock Scenario		1	3	2	4
Microalgae	Genus*	*Scenedesmus*	*Chlorella*	*Scenedesmus*	*Chlorella*
Co-substrate		no	no	Primary Sludge	
SRT	days	50-100	100	100	
HRT	days	15-50	30	30	
OLR	gVS·L-1·d-1	0.2-0.4	0.4	0.5**	

*Identified by microscopic counts of phytoplankton cells in the MPBR harvested biomass
**Resulting OLR of mixing 62% primary sludge and 38% microalgae biomass (based on gVS content).

The digester was first operated for 20 months at different SRT conditions: 50, 70 and 100 days. During these months, the HRT was set at 50 days (for 50 and 70 days SRT) and later at 15 days (for 70 and 100 days) to increase the OLR of the system from 0.2 to 0.4 $gCOD \cdot L^{-1} \cdot d^{-1}$. The AnMBR co-digester started running in parallel to the AnMBR digester after 20 months, fed with the same microalgae feedstock than the AnMBR digester plus the primary sludge. The SRT of the co-digester was fixed at 100 days SRT, as it was optimized in the previous AnMBR digester performance. Both AnMBRs were running in parallel for additional 12 months.

7.2.2. Feedstock sources

Microalgae and primary sludge were obtained from a membrane photobioreactor pilot plant (MPBR) and a primary settler respectively, both located in the municipal WWTP "Cuenca del Carraixet" (Valencia, Spain). The MPBR pilot plant is used to remove nutrients from the anaerobic effluent of an AnMBR pilot plant treating sewage (González-Camejo *et al.*, 2019). The experimental work of this research has lasted almost three years (32 months), in which *Scenedesmus* and *Chlorella* have separately

dominated the MPBR culture. According to microscopic observation and quantification (Pachés *et al.,* 2012), during the first 24 months, more than 90% of the phytoplankton observed in the MPBR were identified as *Scenedesmus* spp. Later, a shift in the microalgae population of the MPBR occurred and instead more than 90% of the cells were *Chlorella* spp. This microalga was dominant in the MPBR for the 8 remaining months.

A cross-flow ultrafiltration hollow-fiber membrane unit (HF 5.0-43-PM500, PURON® Koch Membrane Systems) was used for microalgae harvesting and concentration to the required values before feeding the AnMBRs to an organic loading rate (OLR) of 0.2-0.4 $gVS \cdot L^{-1} \cdot d^{-1}$ (see Table 7.1). Microalgae feedstock was prepared in a single batch for both systems and then adjusted to the different concentrations for single- or co-digestion. The primary sludge was collected from the gravity thickener, sieved through an aperture of 0.5 mm sieve and diluted to 22.8 $gCOD \cdot L^{-1}$ to feed the AnMBR co-digester according to Table 7.1 OLR conditions (62%-38% proportion of primary sludge and microalgae based on gVS determination). The physicochemical characterization of feedstock samples was performed according to APHA (2012) standard procedures. Feedstock sources were separately stored at 4°C (for no longer than 3 weeks) to preserve its characteristics and avoid degradation.

7.2.3. Performance analysis

Physicochemical analysis and biogas production were carried out per triplicate and three times a week as in a previous study (Zamorano-López *et al.,* 2019a). At least the data retrieved during three pseudo-steady state weeks were considered to calculate the methane yield, the biodegradability, the solids content of the system (in terms of total suspended solids, TSS) and the total COD (TCOD). The methane yield was calculated on a COD basis, considering the COD of the methane produced and measured in the biogas over the total influent COD associated to each feedstock scenario. The biodegradability of the system was thus calculated on this basis using the theoretical potential of 350 $mLCH_4 \cdot gCOD_{inf}^{-1}$ (TMP 0ºC, 1 atm) and

expressed as the percentage of the biomethanization achieved for each feedstock scenario.

7.2.4. Sample collection for microbial ecology analysis

Digestate samples were extracted from each AnMBR during the different pseudo-steady state periods achieved for the different combinations of operational parameters applied to each AnMBR (Table 7.1). Since the pseudo steady state was reached before each biomass collection point, samples can be considered biological replicates for each microalgae mono- and co-digestion scenario evaluated. Under each period, methane yield, COD and TSS of the digestate were determined in each AnMBR.

All samples were frozen at -20°C before the nucleic acid extraction. At least two samples were collected for each AnMBR experimental period regardless of the inoculum. In total, 13 samples were collected from the digester, whereas 9 samples were extracted from the co-digester. Co-digester samples were extracted in duplicate from the main tank and the reservoir tank included in the AnMBR co-digester set-up (Figure 7.6b). Two extra samples were also stored from the reservoir tank at days 124 and 170. Hence, 33 samples were used in total in this study for microbial analysis.

7.2.5. Nucleic acid extraction, 16S rRNA gene library preparation and amplicon sequencing

Following the procedures from Zamorano-López *et al.* (2019) the nucleic acids were extracted from each sample and frozen at -20°C before their submission to the sequencing service of the *Fundación para el Fomento de la Investigación Sanitaria y Biomédica de la Comunitat Valenciana* (FISABIO, Valencia, Spain). Primers targeting the v3 to v4 region of the 16S rRNA gene were used for library preparation. The sequencing run was performed in a 2x300 bp paired-end run using an Illumina Miseq sequencer and v3 reagent kit. The raw results can be found in the Sequence Reads Archive (SRA) repository from the NCBI platform: bioproject PRJNA434206, accession numbers SAMN11567542-50 (co-

digester), SAMN11567551-63 (digester) and SAMN11567566-76 (reservoir).

7.2.6. Diversity analysis

The sequences retrieved from the Illumina amplicon sequencing approach were analyzed as in previous studies (see Zamorano-López *et al.*, 2019). Different Operational Taxonomic Units ($OTU_{0.97}$) were defined at a 3% dissimilarity in an open-reference cluster step using QIIME. The weighted UniFrac distance was estimated in all samples to explore the beta-diversity. The richness estimators chao1 and PD whole tree, jointly with the number of $OTU_{0.97}$ observed and the simpson evenness (simpson_e) index were used to analyze the alpha-diversity of the bioreactor extracted samples. Biom resulting table from QIIME containing the $OTU_{0.97}$ composition and taxonomic assignments according to SILVA v128 release was exported to further analyze the microbial community.

7.2.7. Biostatistics

A single-factor ANOVA test ($p<0.05$) was used over biodegradability values calculated from the methane yield achieved in each scenario, being the null hypothesis that all scenarios reached similar biodegradabilities. All biostatistics analysis was performed using R-studio (v.3.2) within vegan and mixomics packages. A principal co-ordinate analysis (PCoA) based on the weighted unifrac distances matrix was used to evaluate the beta-diversity of the different samples collected from both AnMBRs. Adonis test over the PCoA results was performed using 999 permutations for feedstock and digester categorical variable clusters. The co-occurrence of the dominant $OTU_{0.97}$ (relative abundance over 2.0%) was explored through a Pearson correlation matrix of the dominant members observed among the 33 samples sequenced. A Partial Least Square Discriminant Analysis (PLS-DA) was performed over all samples (digester, co-digester, and reservoir) to explore the effect of the primary sludge addition over the AnMBRs populations. This statistical analysis allows extracting the most discriminant $OTU_{0.97}$ among a group of samples and their major association with any of the two AnMBR systems studied here.

7.3.Results and discussion

7.3.1. 16S rRNA sequencing data analysis and alpha-diversity measurements

The 16S rRNA gene amplicon sequencing approach resulted in a total of 1,431,467 raw sequences that after downstream analysis with high-quality settings resulted in an average of 57,409 clean sequences per sample. After rarefaction to the minimum value of clean sequences observed in the dataset (27,647) different alpha diversity estimators were extracted (Table 7.2). To compare these values, only samples taken under the same SRT in each AnMBR scenario were considered, since this parameter can strongly enhance species richness and diversity in anaerobic systems with high solids retention capacity such as the AnMBR.

Table 7.2. Mean and standard deviation alpha diversity estimator values from samples retrieved during the different feedstock scenarios evaluated in the two AnMBRs.

	n*	**chao1**	**PD_whole_tree**	**observed_otus**	**simpson_e**
Scenedesmus	3	6023±1707	124±21	3358±1130	0.017±0.005
Chlorella	2	7075±0	152±0	4150±0	0.041±0
Scenedesmus +Primary Sludge	2	6903±298	149±5	4001±258	0.030±0.019
Chlorella +Primary Sludge	3	6817±298	148±5	3927±258	0.024±0.019

*number of samples used to compare the alpha diversity of the AnMBR samples.

The highest diversity was found in the samples taken during *Chlorella* digestion: 4150 observed OTUs. This scenario also presented the highest diversity in terms of non-detected OTUs, which are estimated through the chao1 index (7075). On the contrary, the *Scenedesmus* scenario presumably had the minimum diversity observed with 3358 OTUs and an estimated 6023 chao1 index value. This could be related to the development of a more specific community for *Scenedesmus* digestion than for *Chlorella digestion*. As it has been reported in the literature, *Scenedesmus* is among the hardest *Chlorophyta* member for direct disruption using microbial communities due to the presence of algaenan (González-Fernández *et al.*, 2018). Although *Chlorella* cell walls are also composed of recalcitrant compounds similar to

chitin (Baudelet *et al.*, 2017), the n-alkaenan composition of algaenan could have a stronger selective pressure effect over microbial communities and therefore decrease AnMBR alpha diversity.

The phylogenetic similarity of each sample can be measured through the PD_whole_tree estimator (Table 7.2). The higher the number of phylogenetic tree branches, the higher the value of PD_whole_tree estimator and thus, this value reveals the existence of more diverse and distant species in each sample. The highest PD_whole_tree values were observed during *Chlorella* digestion, again suggesting that this was the more diverse feedstock scenario of the four studied. Between the two co-digestion scenarios, slight differences were observed in the three indexes (observed_otus, chao1, and PD_whole_tree). This could be related to the higher presence of microbial groups with wider metabolic capacities in the digester when both substrates were present than when it was only fed with microalgae.

The evenness measurement retrieved for each scenario (see simpson evenness index in Table 7.2) reflected that the changes in the relative abundance patterns of the observed OTUs were more dynamic in the co-digestion scenarios than when only microalgae were digested. It should be noticed that from an ecological perspective, the addition of a co-substrate which has a certain microbial diversity can enhance richness and evenness diversity due to the presence of minor and rare groups that might not be active in the anaerobic system but are though retained. Related to this, Chen *et al.* (2019) observed higher diversity in the primary sludge than in the anaerobic digester samples. Thus, primary sludge could also have enhanced evenness in the AnMBR co-digester in this work. Interestingly, Greses *et al.* (2017) pointed out that despite a shared bacterial diversity of 32% between microalgae feedstock and anaerobic digester samples, the resulting communities established in the microalgae digester were significantly different from the influent. Consequently, the influence of diversity-rich feedstock, especially in presence of anaerobic microorganisms (like it occurs in the primary sludge), over anaerobic digestion communities should be

carefully explored in bioreactor configurations such as the AnMBR. In this system, the use of ultrafiltration membranes enhances the retention of niche and biofouling-related microorganisms (Robles *et al.,* 2018; Skouteris *et al.,* 2012). Furthermore, high solids retention capacity enhances microbial persistence resulting in microbial communities with high diversity and richness, according to 16S rRNA/rRNA gene sequencing results (Mansfeldt *et al.,* 2019). Hence, further research would be required to identify the feedstock-incoming microorganisms that are later retained in the system to improve our measurements of richness and evenness diversity in high solids retention systems such as the AnMBR.

7.3.2. Beta diversity analysis reveals different structures of microalgae-degrading communities in the AnMBRs

According to the beta-diversity analysis performed through PCoA over the weighted unifrac distance matrix, there are different structures among samples depending on the microalgae biomass used as feedstock and the addition or not of a co-substrate (*e.g.* primary sludge). The first component of the PCoA explains the 38% of the differences between the samples that were collected from the digester when the primary sludge was added or not added. The second component explains 27% of the variability between samples and especially remarks a change in the structure of co-digester samples (Figure 7.1).

As shown in Figure 7.1, samples were categorized according to the digester and the feedstock. For the first categorical variable, two clusters were revealed by the Adonis test (digester, $p<0.001$). Hence, the microbial structure of the co-digester and its reservoir was consistent in between but differed from the microbial structure of the digester samples. The second categorical variable used in the Adonis test revealed the existence of three clusters (feedstock, $p<0.011$), although four feedstock scenarios were analyzed in the present study. Thus, the differences in the microbial community structures of both AnMBRs should be attributed to the addition or not of a co-substrate and not to the species of microalgae fed to the reactor. The microbial structure in the digester did not shift significantly when

feeding *Scenedesmus* or *Chlorella.* The change in the microalgae did not either disturb the microbial structure of the co-digester since the co-digester early stages samples are grouped with the *Scenedesmus* and primary sludge scenario samples (see top left corner samples in Figure 7.1). Finally, the differences among the digester samples were related to the effect of the SRT over the microbial population and the acclimation trend of the biomass, as previously mentioned.

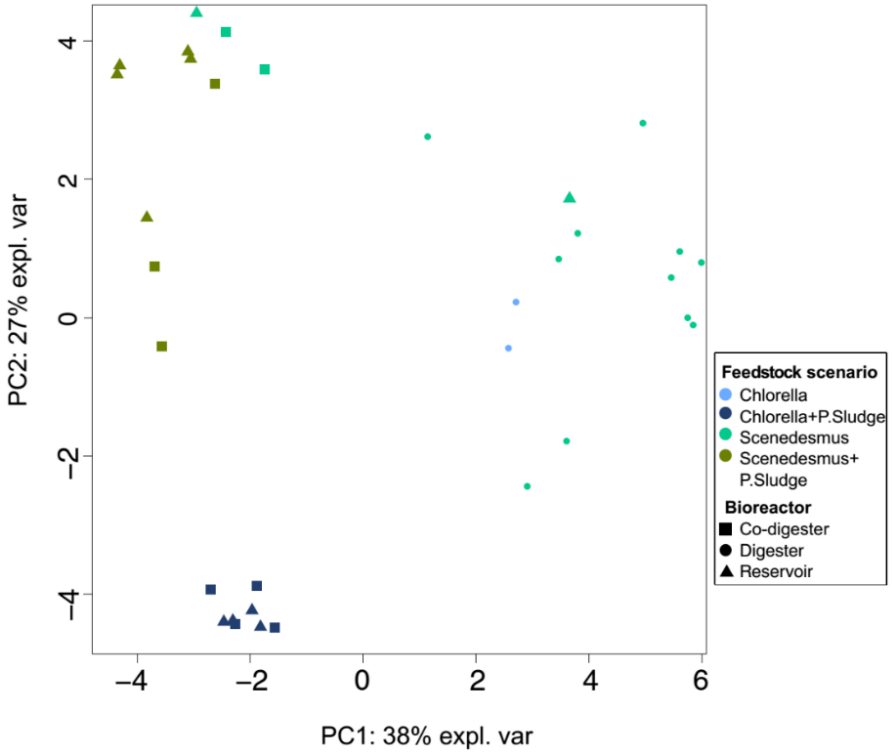

Figure 7.1. Principal Co-ordinate Analysis (PCoA) showing differences in the feedstock composition. Adonis tests revealed three feedstock ($p<0.011$) and two digester clusters ($p<0.001$). Abbreviations in the feedstock legend refer to: *Chlorella* (Chlo), *Chlorella*+Primary Sludge (Chlo-Ps), *Scenedesmus* (Sc) and *Scenedesmus*+Primary Sludge (Sc-Ps) scenarios. The digester legend shows the AnMBR codigester (squares), AnMBR digester (circles) and the reservoir from AnMBR codigester (triangle) samples.

The proximity between the samples collected when digesting *Scenedesmus* or *Chlorella* observed through the PCoA (Figure 7.1) suggest the potential use of the same anaerobic biomass to degrade these two algae.

This is a remarkable fact and highlights the potential use of this acclimated biomass in microalgae-based bioenergy recovery processes. This concept which is based on a circular economy requires low-cost stages of microalgae disruption. An attractive strategy is to use these acclimated microbial communities as hydrolytic biomass sources and convert microalgae into biomethane through anaerobic digestion. Both *Chlorophyta* belonging genera are commonly found in freshwater and spontaneously grow over sewage-treated effluents (Garrido-Cárdenas *et al.*, 2018). Hence, the findings here reported support the use of this biological strategy in a loop-system combining microalgae cultivation using anaerobically treated sewage-effluents, biomass harvesting and their further conversion into energy.

7.3.1. Combining feedstock acclimation and high SRT operation to promote microalgae degrading microorganisms

During SRT acclimation from 50 days up to 100 days in the digester AnMBR, slow-growing hydrolytic microorganisms were selected allowing the degradation of raw microalgae with remarkable methane yields (Table 7.3). Figure 7.2 shows the relative abundances calculated at the phylum level from the $OTU_{0.97}$ compositions among samples of the AnMBR digester. The changes in the patterns reveal the effect of SRT over microbial composition.

Table 7.3. Characterization of the AnMBR feedstock scenarios during their pseudo-steady state in terms of mean and standard deviation values of methane yield, total COD (TCOD) and total suspended solids (TSS) in the AnMBR digestate.

		AnMBR scenarios			
		1	*2*	*3*	*4*
Methane yield	$mLCH_4 \cdot gCOD_{inf}^{-1}$	172±7	241±18	214±7	228±3
Biodegradability*	%	49±2	69±5	61±2	65±1
TCOD	$gCOD \cdot L^{-1}$	6143±64	17524±73	6041±64	16134±80
TSS	$gTSS \cdot L^{-1}$	4280±146	12739±129	4406±232	12099±259

AnMBR influent scenarios: (1) *Scenedesmus*, (2) *Scenedesmus*+Primary Sludge, (3) *Chlorella* and (4) *Chlorella*+Primary Sludge (n≥3 weeks). *Biodegradability was calculated according to a biomethanization potential of 350 $mLCH_4 \cdot gCOD_{inf}^{-1}$ according to Greses *et al.* 2017.

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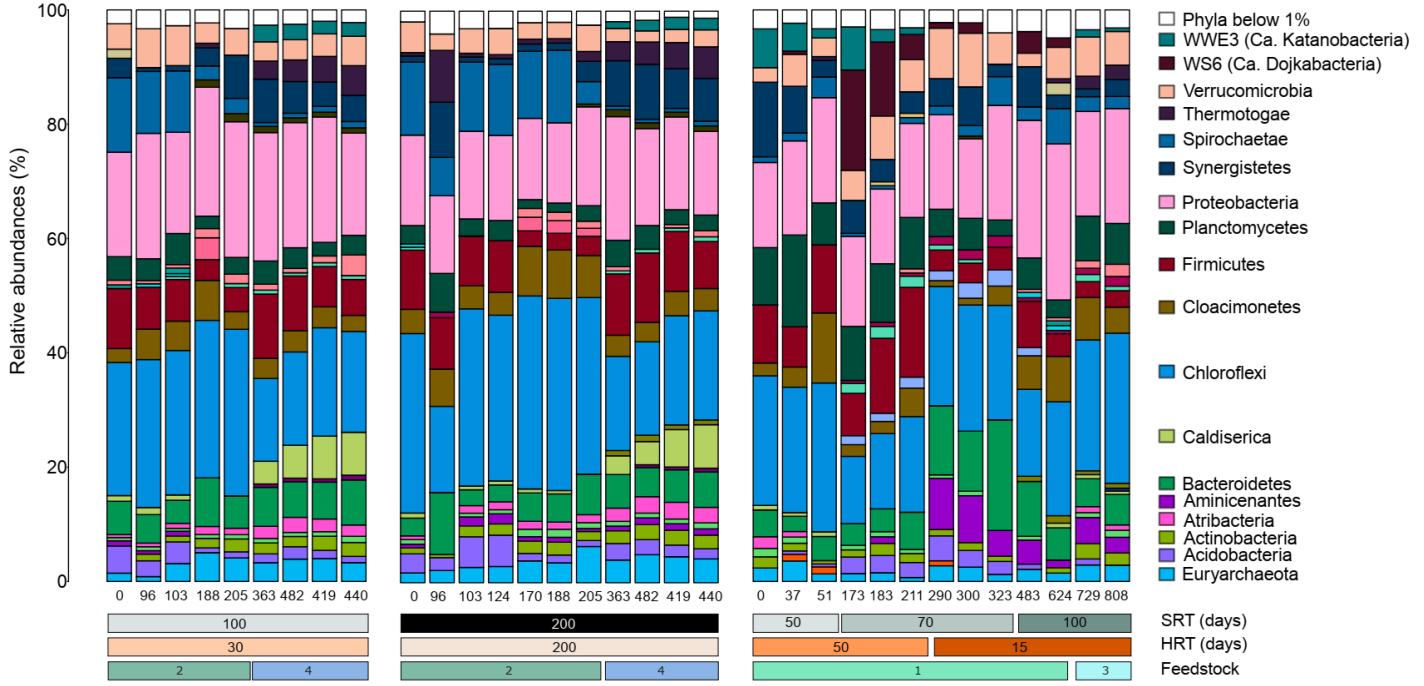

Figure 7.2. Relative abundance barplots of the different dominant phyla (threshold value 1%) observed in during the experimental work. Bioreactor barplot blocks belong to AnMBR co-digester (left), reservoir tank (center) and AnMBR digester (right). Operational conditions are shown in horizontal bars: solids retention time (SRT), hydraulic retention time (HRT) and feedstock. Feedstock scenario labels are as follows: *Scenedesmus* (1), *Scenedesmus*+Primary Sludge (2), *Chlorella* (3), *Chlorella*+Primary Sludge (4). Further details can be found in supplementary Tables 7.4-7.8).

During the operation at the lowest SRT (50 days) the dominant phyla observed were: 23.5% *Chloroflexi*, 16.6% *Proteobacteria*, 11.1% *Planctomycetes* and 9.7% *Firmicutes* (Table 7.8, supplementary). These four groups were present during the whole experimental period and are common groups of anaerobic digesters, as shown in different studies of full-scale systems (Calusinska *et al.*, 2018b; De Vrieze *et al.*, 2018) and also in microalgae digesters (Córdova *et al.*, 2018; Greses *et al.*, 2018, 2017; Klassen *et al.*, 2017; Sanz *et al.*, 2017) or co-digesters (Li *et al.*, 2017a; 2017b). However, their relative abundances changed under different SRT operations as other microbial groups like *Bacteroidetes, Cloacimonetes, Spirochaetes, Aminicenantes* and Candidatus *Dojkabacteria* (WS6 phylum) thrived in the system and co-existed with the previous phyla.

The operation at 70 days SRT with an HRT of 50 days was characterized by the remarkable presence of the Ca. *Dojkabacteria* (14.8%, see Table 7.8). This novel group is poorly described and none of the belonging members has been isolated yet. Their early identification by Dojka *et al.* (2000) using culture-independent approaches (16S rRNA gene cloning) suggested their importance in organic-rich environmental anaerobic niches. Up to the present date and the knowledge of the authors of this manuscript, no other studies have clearly described their function in anaerobic digesters for microalgae conversion into energy. Interestingly, Qiao *et al.* (2013) observed Ca. *Dojkabacteria* during anaerobic digestion of corn straw. According to more recent metagenomic findings Ca. *Dojkabacteria* related OTUs have xylan disruption capacity (Solden *et al.*, 2016). This sugar is commonly observed in *Chlorophyta* cell walls (Baudelet *et al.*, 2017; Domozych, 2014) and hence, the role of this phylum in microalgae degradation could be suggested from these findings.

However, Ca. *Dojkabacteria* presence decreased in the AnMBR after changing the HRT from 50 to 15 days. An antagonist response was observed for *Firmicutes* phylum, which was favored during increased SRT operation at 70 days, reaching relative abundance values up to 12.5% (sample 211, Figure 7.2). Different members of *Firmicutes* are commonly reported in

complex polysaccharide anaerobic degradation since they can release enzymes to the environment and disrupt complex molecules (Calusinska *et al.,* 2018b; Cheng *et al.,* 2014). The *Firmicutes* phylum decreased in terms of relative abundance after the HRT reduction from 50 to 15 days, suggesting that other microbial groups have a higher affinity for the substrates and thrived in the AnMBR. Despite the maintenance of an SRT of 70 days, the reduction of the HRT increases the organic loading rate of the system and reduces the contact time in between the soluble phase and the microorganisms. Thus, lower HRT can affect the mass transference of the system and enhance microbial groups with lower specific rates of substrate utilization.

During operation at high 15 days HRT and high SRT the relative abundances of *Bacteroidetes* and *Aminicenantes* phyla increased at 100 days SRT. Both groups remained in the AnMBR digester during operation at 70 days SRT, although their relative abundance values were lower over time and especially at the end of *Scenedesmus* digestion (samples 483 and 624, Figure 7.2). Then, for *Chlorella* digestion also at high SRT of 100 days and low HRT of 15 days, changes in the phyla profiles were observed. Consequently, the relative abundances of both *Bacteroidetes* and *Aminicenantes* were lower for *Chlorella* digestion scenario than for *Scenedesmus.* Both phyla have been related to the core of wastewater anaerobic digestion systems in a recent study performed over twenty years targeting the 16S rRNA gene (Calusinska *et al.,* 2018b). The role of *Bacteroidetes* in the present work could be more heterogeneous, as different members related to this phylum are involved in both polysaccharide and peptide degradation. Indeed, *Bacteroidetes* has been remarked as a key phylum continuous raw microalgae digestion for methane production (Klassen *et al.,* 2017). On the other hand, Farag *et al.* (2014) early suggested the wide potential metabolic implication of *Aminicenantes* in anaerobic environments. However, little is known about this group as none of the representative members of this has been isolated yet as a pure culture, but recent findings suggest their importance in hydrogen and acetate production

after saccharolytic degradation (Kadnikov *et al.,* 2019). Hence, they could play an important role during microalgae degradation at high SRT as methanogenic substrate donors.

According to these results, a robust long-time acclimation of the mesophilic inoculum used in the digester resulted in an enrichment of potential microalgae degraders from the *Chloroflexi, Proteobacteria, Bacteroidetes* and *Aminicenantes* phyla that were retained in the system through membrane operation even under different SRT conditions. Hence, this acclimated community could be inoculated in another anaerobic system coupled to future WRRFs to produce bioenergy from sewage in an anaerobic-microalgae loop technology.

7.3.2. A 57% shared microbial core between the four feedstock scenarios tested for microalgae and primary sludge biomethanization

The microbial core for microalgae biomethanization was elucidated in this study through a Venn diagram (Figure 7.3). A total number of 578 $OTU_{0.97}$ were shared between the AnMBR operated under different scenarios, corresponding to a shared-diversity of 57%. Also, the Venn diagram revealed the presence of unique $OTU_{0.97}$ in the four scenarios. The scenario with the highest number of unique members was *Chlorella* and Primary Sludge (131 $OTU_{0.97}$). The remaining scenarios had 103, 109 and 92 specific $OTU_{0.97}$ (*Scenedesmus, Scenedesmus* and Primary Sludge and *Chlorella*, respectively) (Figure 7.3). The small difference between the digestion and co-digestion scenario for *Scenedesmus* contrasts with the high difference in terms of unique $OTU_{0.97}$ of *Chlorella* digestion and co-digestion, which showed the lowest and highest value of unique members. These findings are similar to the alpha diversity analysis results since *Scenedesmus* scenarios had a higher specificity than *Chlorella* scenarios. However, any of the unique $OTU_{0.97}$ were not present in a relative abundance of over 0.7% in any sample. Hence, the presence of specific members in each different scenario might not be as important as the persistence of a microbial core of 578 $OTU_{0.97}$ that are shared in between the four scenarios.

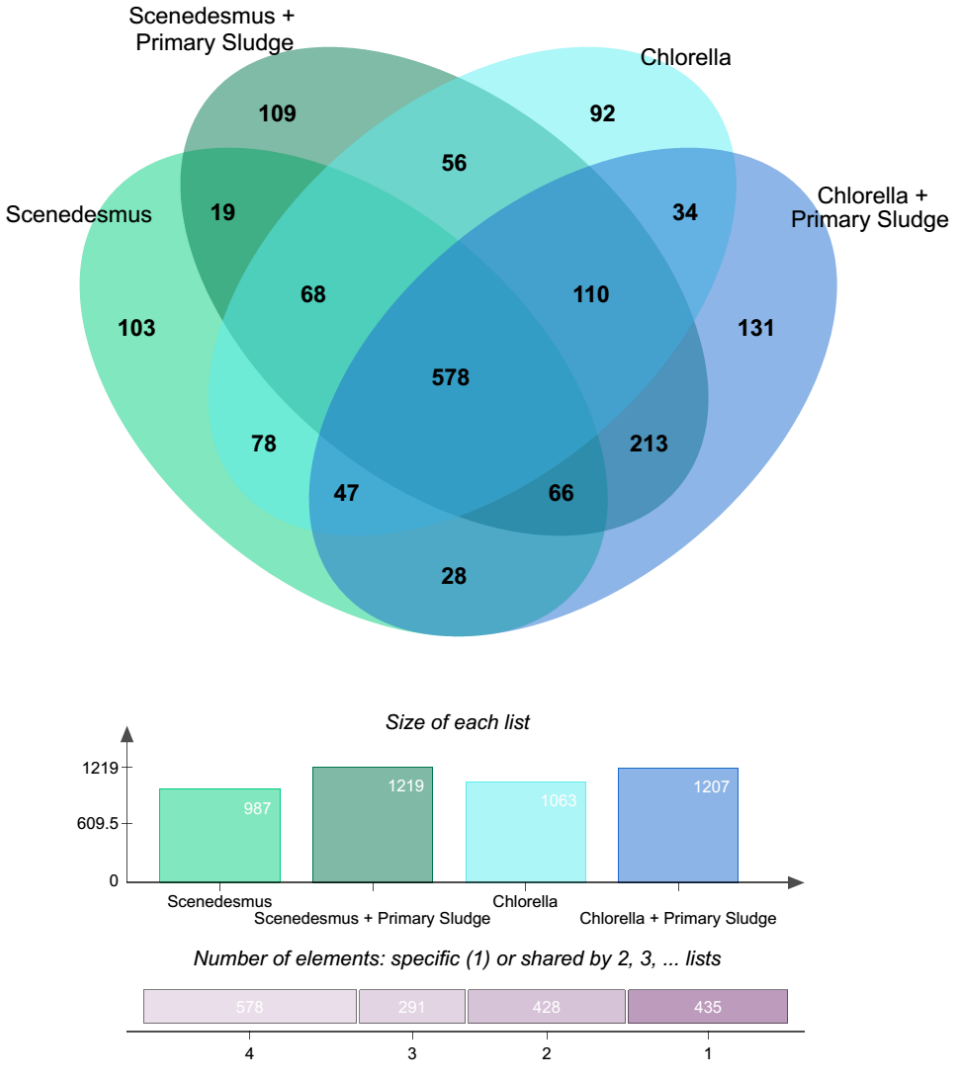

Figure 7.3. Venn diagram showing the core OTUs identified for each feedstock analyzed in the digesters. Venn areas showing shared and non-shared OTUs (top) and size in terms of OTUs observed for each substrate scenario (center). Horizontal bars (bottom) summarize the shared (4) or non-shared (1) OTUs between all scenarios (4) and the sum of the different combinations of OTUs shared by 2 or 3 scenarios (horizontal bar labels 2 and 3, respectively).

This microbial core has been defined in terms of dominant relative abundances. However, further research is needed to develop future methodologies for monitoring the dynamics of these groups in anaerobic systems producing energy from microalgae. Since microalgae degradation is complex from a metabolic perspective due to the heterogeneous composition of microalgae cell walls (Baudelet *et al.,* 2017), targeting the members of the microalgae-degrading microbial core could be an effective strategy to monitor microalgae digesters. A future necessary step would be the design of specific probes or oligonucleotides that can target the rRNA and provide the activity levels of these groups. Besides, qPCR approaches or 16S rRNA gene sequencing coupled to flow-cytometry sorting systems (Rinke, 2018; Wang *et al.,* 2010) or including a spike control (Stämmler *et al.,* 2016) could provide absolute measurements of these relevant microorganisms for bioenergy production. Besides these aspects, further information about the composition of the feedstock could positively contribute to our understanding of the metabolic implications of the core microorganisms. Hence, further studies should explore the content of carbohydrates, proteins and lipids and contribute to metabolic flux studies (Weinrich *et al.,* 2019). Future studies should jointly evaluate the effect of the operational parameters of the systems and the metabolic interactions between microorganisms over the microbial cores of anaerobic digestion. Towards the development of future microbial-based models of anaerobic digestion of complex feedstocks that are produced in WRRF, this effort should be considered since microbial communities cannot be longer overstated (Widder *et al.,* 2016).

7.3.3. Co-digestion scenarios reached higher biomethanization and higher relative abundance of Anaerolineaceae, Smithella and Methanosaeta

In the present work, another AnMBR was run in parallel using the same microalgae biomass plus primary sludge collected from the WRRF primary settler. Similar communities might be established when treating the same feedstock sources, as a result of the stabilization of a microbial core in

biogas reactors (Zuopeng *et al.*, 2019). The intergroup differences observed among the four feedstock scenarios were significantly different ($p<0.05$) according to the ANOVA test over the biodegradabilities calculated from the methane yield (Table 7.3). The highest biomethanization values were indeed observed for *Scenedesmus* and *Chlorella* co-digestion scenarios: 241 and 228 $mLCH_4 \cdot gCOD_{inf}^{-1}$ respectively (Table 7.3). Hence, the type of microalgae might have a higher impact over the biomethanization only during digestion and not during co-digestion. Related to this, a recent study from Solé-Bundó *et al.* (2019) reported a methane yield of 330 $mLCH_4 \cdot gVS^{-1}$ when digesting *Chlorella* with primary sludge. In our case the digestion of *Chlorella* produced 417 $mLCH_4 \cdot gVS^{-1}$ and the co-digestion 441 $mLCH_4 \cdot gVS^{-1}$, according to an average content of 50% proteins, 23% carbohydrates and 18% lipids (reference values reported by Klassen *et al.* 2016). Our increment in the biomethanization after co-digestion was lower than the increment observed by Solé-Bundó *et al.* (2019). However, our methane yields seem to be higher. This could be related to the long acclimation of the biomass to *Chlorella.* The SRT and HRT conditions applied in our study combine a high SRT with a short-decoupled HRT, whereas the study from Solé-Bundó *et al.* (2019) applied a fixed SRT of 20 days (same HRT). As reported by Greses *et al.* (2017), the longer the SRT, the higher potential for microalgae disruption driven by microorganisms that might have low kinetics.

Figure 7.4 shows the most abundant $OTU_{0.97}$ found in the microbial core. *Smithella* genus, a syntroph belonging to the *Syntrophobacterales*, was predominantly observed in the co-digesters, coinciding its highest values within the highest presence of *Methanosaeta* (order *Methanosarcinales*). The simultaneous presence of *Smithella* and *Methanosaeta* was observed in our study with a 0.45 Pearson correlation value. The microalgae digesters, compared to the co-digesters, presented lower abundance of *Methanosaeta* (1.3% when digesting *Scenedesmus* and 0.6% with *Chlorella*). *Smithella* was detected also at very low values (4.8% and 0.6%, for *Scenedesmus* and *Chlorella*, respectively). This could be related to the less balanced scenario

for methane production as the C:N ratio in the digester was lower than in the co-digester. As reported by Leng *et al.* (2018), *Methanosaeta* and *Smithella* are common genera in anaerobic digestion processes and play an important role during methane production after fatty acid conversion into a more reduced form *i.e.* acetate. The degradation of propionate generated by *Smithella* depends on the hydrogen consumption by other microorganisms. In our case, this role could be related to the genus *Defluviitoga*, which has been recently related to mechanisms of electron transport chains including hydrogen scavenging (Fontana *et al.* 2018). *Defluviitoga* had a positive correlation with both *Smithella* and *Methanosaeta* (Pearson correlation coefficients of 0.33 and 0.43, correspondingly). However, other non-dominant $OTU_{0.97}$ that were not considered for co-occurrence analysis could have also contributed to uptake hydrogen and allowed methane production through the *Smithella* pathway.

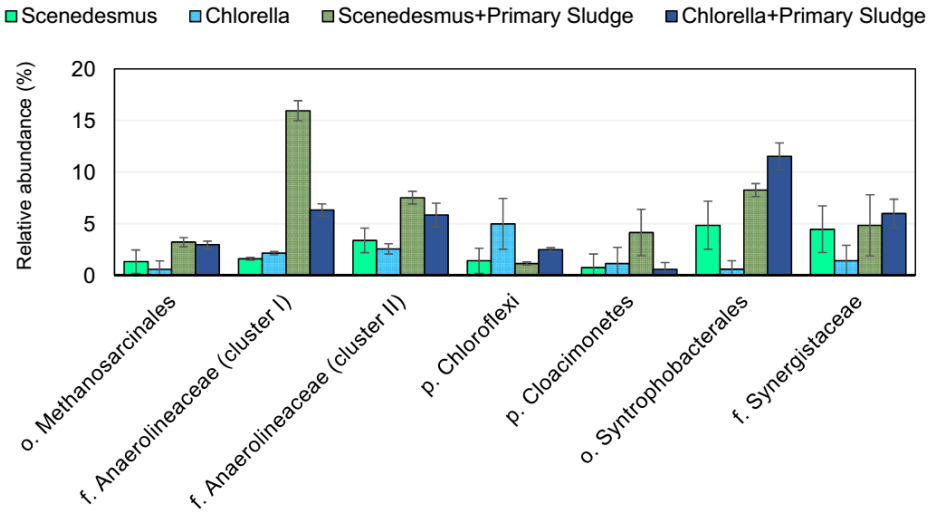

Figure 7.4. Relative abundances of the microbial core for microalgae biomethanization elucidated from 16S rRNA gene sequencing. The mean and standard deviation have been calculated from the samples that belong to the same SRT conditions among the different scenarios ($n \geq 2$).

Anaerolineaceae clusters I and II were observed in higher relative abundances in the co-digestion scenarios (Figure 7.4). The abundance of

these groups was positively correlated with *Methanosaeta* relative abundance (Pearson coefficient of 0.25). According to the review from McIlroy *et al.* (2017), all isolated members of this family are donors of acetate after fermentation of carbohydrates. Also, this family has been proposed as biological disrupters of microalgae (Greses *et al.*, 2017; Sanz *et al.*, 2017) besides macroalgae (Zou *et al.*, 2018) and would be involved in the production of other fermentation products such as lactate, hydrogen, and formate. Interestingly, the cells of the microorganisms belonging to this group are filamentous types. A recent study from Bovio *et al.* (2019) supports their importance in granule generation in Up-flow Anaerobic Sludge Blanket (UASB) systems. This is a key capacity also during biofouling and cake formation processes in AnMBR systems that could explain the dominance of *Anaerolineaceae* in the present study. Moreover, as reported by McIlroy *et al.* (2017), *Anaerolineaceae* and *Methanosaeta* are commonly associated forming a complex filamentous network. If this group was major donors of acetate to *Methanosaeta* in this work, the association in a "spaghetti-like" structure of *Anaerolineaceae* and the aceticlastic methanogen could have promoted the metabolites transfer flux between both groups, resulting in high methane production rates.

In summary, our findings suggest the relevant role of propionate production and further reduction during the digestion of microalgae with and without co-substrate. The higher detection of potential syntrophs such as *Smithella* and *Defluviitoga* during co-digestion might be related to the favorable effect of the addition of an extra carbon source to the AnMBR. Since microalgae composition is less heterogeneous than primary sludge, metabolic pathways might tend to be more specific with higher reliance on fermentation of sugars into acetate or on amino acid fermentation after protein lysis. Besides, the synergies promoted by the addition of primary sludge would be reflected in the diversity of intermediate steps before methane production such as the propionate-depending *Smithella* pathway.

7.3.4. PLS-DA analysis to find differences between microalgae digestion and co-digestion with primary sludge from relative abundance magnitude

All $OTU_{0.97}$ including minor and rare groups were considered for PLS-DA model construction. As detailed in the methods Section 7.2.7, those groups at very low relative abundances are removed during downstream sequencing analysis. However, several groups that are in relative abundances values below 1% but might play an important functional and ecological role in complex microbial networks (Rivière *et al.,* 2009). Interestingly, these groups might be the most discriminants of each microbial structure observed during microalgae digestion and co-digestion with primary sludge due to their presence or absence.

Figure 7.5 shows the results of the fitted PLS-DA regression model. As can be seen in this figure the samples from the microalgae digestion are well separated from the samples from co-digestion. To elucidate the most discriminant groups between both digestion substrates, the variable importance in the projection (VIP) was calculated. The first 30 microbial members sorted by the highest VIP value retrieved from PLS-DA are shown in Figure 7.9. Genera belonging to Actinobacteria, *Atribacteria, Chloroflexi, Cloacimonetes, Firmicutes, Proteobacteria, Spirochaetae, Verrucomicrobia* (*Bacteria*) and WSA2 (*Archaea*) were among the most discriminant ones. Some of them are classified inside of the dominant phyla observed in both AnMBRs (Figure 7.2). However, others like Ca. *Caldatribacterium* (phylum *Atribacteria*) are detected at very low abundances (1-2%) but were highly discriminating between samples. According to Dodsworth *et al.* (2014), this group can perform saccharolytic fermentation from cellulosic as well as hemicellulosic substrates. Since cellulose is present in common WTTP primary sludge stream in about 30-50% of the influent suspended solids (Crutchik *et al.,* 2018), the thrive of this bacteria group during co-digestion but not when only microalgae was digested could be related to the higher presence of this complex polysaccharide in the feedstock. *Treponema*, a *Spirochaetae* member, was

also among the most discriminant and found only in the samples from co-digestion. The presence of this group was associated with a co-digestion study of sewage sludge and food waste (Cheng *et al.*, 2014). Besides, the saccharolytic capacity of *Treponema* might explain their presence in this work and other microalgae degrading bioreactors (Klassen *et al.*, 2016; Sanz *et al.*, 2017). Nevertheless, future analysis with complementary approaches to amplicon sequencing such as proteomics would be needed to understand the complete metabolic implication of these groups and elucidate their link to primary sludge digestion or microalgae degradation.

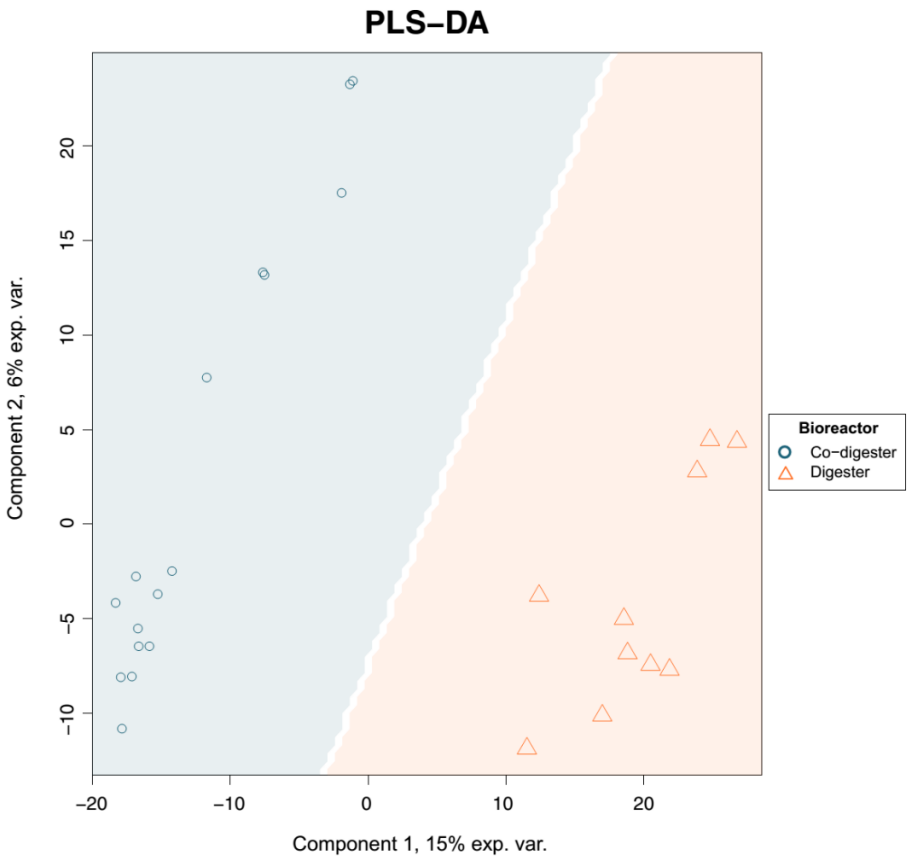

Figure 7.5. Partial Least Square Discriminant Analysis (PLS-DA) according to the co-digestion or digestion process. The background prediction area has been calculated according to the Mahalanobis distance and therefore considers the covariance between the two X-variates retrieved from the PLS-DA model.

7.3.5. Ecological implications of complex and diversity richness during raw feedstock anaerobic digestion and future research needs

The use of microbial-rich biomass sources as co-substrate might present a drawback when using biological strategies to convert microalgae into biogas. The primary sludge strongly shaped the microbial communities in the co-digester as shown in the PCoA (Figure 7.1). From a microbial ecology perspective, this could also be partially related to the accumulation of co-substrate incoming microorganisms and groups entering the system might be viable during microalgae co-digestion. Primary sludge has a high species richness. Although its diversity has not been evaluated on its own in the present study and is rarely evaluated in similar studies, Ju *et al.* (2017) observed 3424 $OTU_{0.97}$ in the primary sludge seed used for their anaerobic digestion trials. However, this study only relied on the microbial characterization through the biomarker 16S rRNA gene and could not, therefore, evaluate the survival of these potential microbial groups present in the influent. Further research using transcriptomic approaches might help to elucidate the activity levels of the microorganisms observed. Since some of the microorganisms are anaerobic and might be acclimated to cellulolytic components present in the primary sludge, they could improve the later digestion of microalgae during the co-digestion.

The present work has demonstrated that a core representing the 57% of the microbial diversity is maintained over time in bioreactors treating microalgae. The maintenance of a core microbiome in anaerobic reactors was reported to be extremely relevant to maintain the functional status (Rivière *et al.,* 2009). Peces *et al.* (2018) reported a convergent diversity after 120 days of continuous operation of four different anaerobic digesters, inoculated with different sources but identically operated to produce biogas from cellulose:casein feedstock. According to these authors, the microbial core contained 78% of the anaerobic digesters diversity. The neutral theory predicts that populations are driven by deterministic factors such as SRT, HRT, and OLR, as it has been demonstrated using different inocula to anaerobically degrade cellulose (Vanwonterghem *et al.,* 2014a). Up to date,

most of the microbial core focused studies have only used the target 16S rRNA gene. Therefore, further research is needed to elucidate the active microbial core, as minor groups might have a relevant role during microalgae digestion. This has been suggested in the present study through application of PLS-DA that remarks the importance of the presence or absence of certain groups to shape microbial structures, despite their low relative abundances. On this basis, RNA-based sequencing (De Vrieze *et al.*, 2018) could facilitate a better profile of key microorganisms during microalgae digestion and especially during co-digestion. The functional profiling of anaerobic communities is a necessary step towards the development of new probes to monitor the wealth of anaerobic digesters from a microbiologist perspective. Also, to retrieve more accurate information in future microbial ecology studies of anaerobic digesters, efforts in targeting the active cells like active cell sorting in flow cytometers and later sequencing (Nakamura *et al.,* 2016) or RNA-based sequencing (De Vrieze *et al.,* 2017) would be required.

Conclusions

A microbial core of 57% shared diversity has been elucidated from four different feedstock scenarios for raw microalgae conversion into biogas. The high presence of several *Anaerolineaceae* members highlights the importance of saccharolytic and peptidic hydrolysis and fermentation. The dominance of *Smithella, Methanosaeta* and *Defluviitoga* suggests the relevant role of syntrophic and methanogenic pathways for bioenergy production from raw microalgae. This association was more effective during co-digestion than when only microalgae was digested, probably because of the composition of primary sludge. Nonetheless, no significant change in the acclimated communities was observed during the microalgae shift from *Scenedesmus* to *Chlorella*. Instead, the microbial core was maintained over time in both AnMBR.

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Authors contributions

NZL: conception and design, analysis and interpretation of the data, drafting of the article, collection and assembly of data. DA: statistical expertise. DA and LB: critical revision of the article for important intellectual content, analysis and interpretation of the data. AS: provision of study materials or patients and obtaining of funding. All authors: final approval of the article

Supplementary data

Supplementary data associated with the present study can be found in the digital version of this manuscript. Supplementary material 1 provides additional figures and tables describing the anaerobic bioreactors, the biomass samples, the relative abundance values retrieved from each sequenced sample and further details about PLS-DA analysis. Supplementary material 2 summarizes the Pearson correlation matrix retrieved from the sequencing data in a sheet and a heatmap plot forms.

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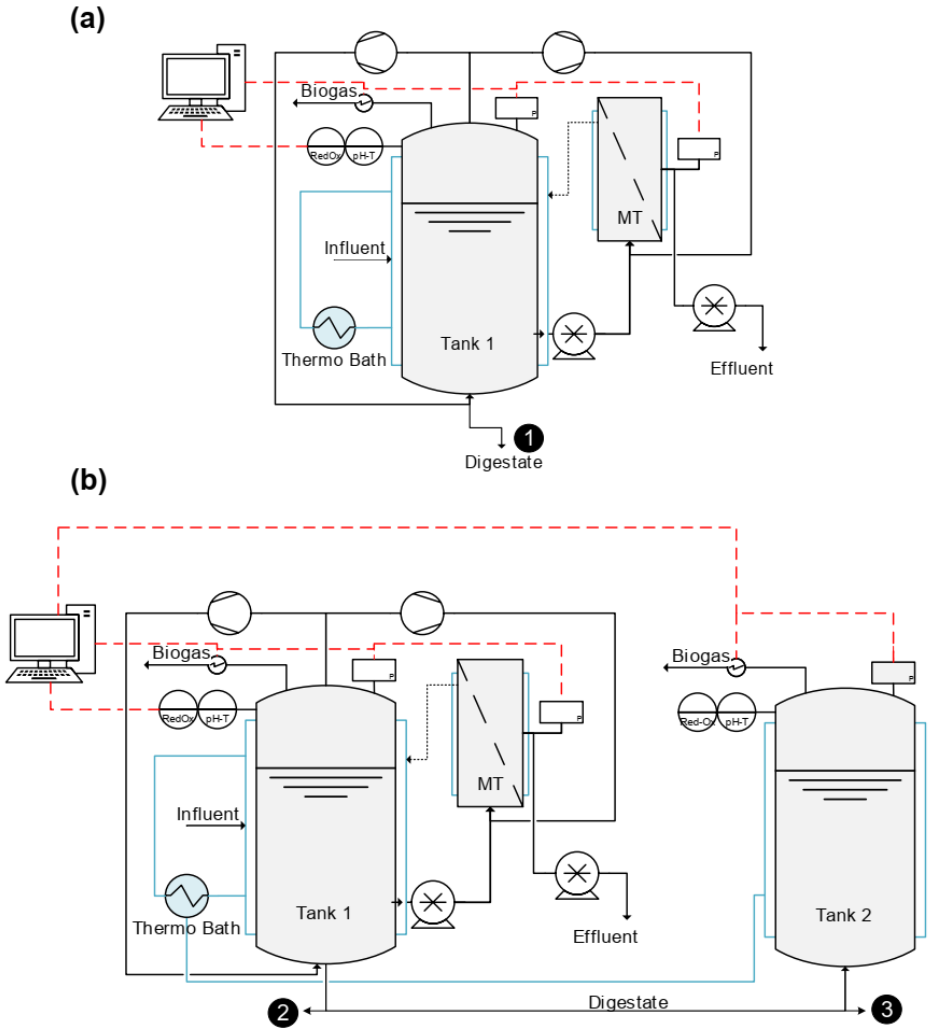

Figure 7.6. Diagrams of the anaerobic systems used in the study with (a) anaerobic membrane bioreactor (AnMBR) configuration and (b) AnMBR plus the reservoir configuration, used for the digestion and co-digestion performances, respectively. The external membrane tank (MT) is coupled to the Tank 1 in both systems to operate in AnMBR configuration. Sampling points are indicated with numbers for AnMBR digester digestate (1), AnMBR co-digester digestate (2) and reservoir digestate (3)

Table 7.4. Summary of the operational conditions corresponding to all the samples analyzed in the study.

#	System*	Sampl. point	Feedstock Scenario**	Days	SRT (d)	HRT (d)	Reactor	T (°C)
1	C	2	2	0	100	30	AnMBR	36
2	C	2	2	96	100	30	AnMBR	36
3	C	2	2	103	100	30	AnMBR	36
4	C	2	2	188	100	30	AnMBR	36
5	C	2	2	205	100	30	AnMBR	36
6	C	2	4	363	100	30	AnMBR	36
7	C	2	4	482	100	30	AnMBR	36
8	C	2	4	419	100	30	AnMBR	36
9	C	2	4	440	100	30	AnMBR	36
10	R	3	2	0	200	200	CSTR	36
11	R	3	2	96	200	200	CSTR	36
12	R	3	2	103	200	200	CSTR	36
13	R	3	2	124	200	200	CSTR	36
14	R	3	2	170	200	200	CSTR	36
15	R	3	2	188	200	200	CSTR	36
16	R	3	2	205	200	200	CSTR	36
17	R	3	4	363	200	200	CSTR	36
18	R	3	4	482	200	200	CSTR	36
19	R	3	4	419	200	200	CSTR	36
20	R	3	4	440	200	200	CSTR	36
21	D	1	1	0	50	50	CSTR	35
22	D	1	1	37	50	50	CSTR	35
23	D	1	1	51	50	50	CSTR	35
24	D	1	1	173	70	50	AnMBR	35
25	D	1	1	183	70	50	AnMBR	35
26	D	1	1	211	70	50	AnMBR	35
27	D	1	1	290	70	15	AnMBR	35
28	D	1	1	300	70	15	AnMBR	35
29	D	1	1	323	70	15	AnMBR	35
30	D	1	1	483	100	15	AnMBR	35
31	D	1	1	624	100	15	AnMBR	35
32	D	1	3	729	100	15	AnMBR	35
33	D	1	3	808	100	15	AnMBR	35

Systems are AnMBR Co-Digester (C), Reservoir (R) and AnMBR Digester (D). **Feedstock scenarios are: Scenedesmus (1), Scenedesmus+Primary Sludge (2), Chlorella (3), Chlorella+Primary Sludge (4).

Table 7.5. Relative abundances summary of the four different mono- and co-digester AnMBR scenarios analyzed. Mean and standard deviation values are shown (n≥2).

		*Feedstock scenario**			
Phylum	***Taxonomic assignment (class, order, family, genus)***	***1***	***2***	***3***	***4***
Euryarchaeota**	*Methanomicrobia; Methanomicrobiales; Methanoregulaceae; Methanolinea*		0.8±1.2		
	Methanomicrobia Methanosarcinales; Methanosaetaceae; Methanosaeta	**1.3±1.1**	**3.2±0.1**	**1.2±0.8**	**2.9±0.4**
Acidobacteria	*c5LKS83 group*	2.9±1			1.0±0.8
Aminicenantes		7±2.3		3.5±1.3	
Armatimonadetes					0.3±0.5
Atribacteria	*Candidatus Caldatribacterium*		1.2±0.2	0.5±0.7	2.2±0.3
Bacteroidetes	*Bacteroidetes vadinHA17*	0.8±0.7		2.0±0.2	0.3±0.5
	Bacteroidia; Bacteroidales; 009E01-B-S-P15;				0.4±0.7
	Bacteroidia; Bacteroidales; Rikenellaceae; S50				0.6±0.7
	Bacteroidia; Bacteroidales; Rikenellaceae; vadinBC27				0.3±0.5
	Sphingobacteriia; Sphingobacteriales; Lentimicrobiaceae	10.1±3.6	3.9±2.5		1.2±0.1
Caldiserica	*Caldisericia; Caldisericales; Caldisericaceae; Caldisericum*				5.7±1.7
Chloroflexi	*Anaerolineae; Anaerolineales; Anaerolineaceae*	1.6±0.1	15.9±1.0	2.1±0.2	6.3±0.6
	Anaerolineae; Anaerolineales; Anaerolineaceae; Leptolinea	1.3±0.2		0.6±0.8	
	Anaerolineae; Anaerolineales; Anaerolineaceae; Longilinea		1.7±0.1	2.0±0.1	0.3±0.6
	Anaerolineae; Anaerolineales; Anaerolineaceae; Pelolinea	5.1±0.6			
	Anaerolineae; Anaerolineales; Anaerolineaceae; Pelolinea	3.7±0.5			
	Anaerolineae; Anaerolineales; Anaerolineaceae	**3.4±1.2**	**7.1±0.1**	**5.3±0.1**	**5.8±1.1**
	Caldilineae; Caldilineales; Caldilineaceae;			3.1±0.2	
	SBR2076	1.4±1.2	1.1±0.1	5.0±2.5	2.5±0.2
	SJA-68			0.5±0.7	
Cloacimonetes	***Candidatus Cloacamonas 1***	**0.7±1.3**	**4.1±2.3**	**1.1±1.6**	**0.6±0.7**
	Candidatus Cloacamonas 2			4.1±1	2.1±0.4
Deferribacteres		2.1±0.7			
Firmicutes	*Clostridia; 8A-2;*				1.0±0.7
	Clostridia; Thermoanaerobacterales; Thermoanaerobacteraceae; Gelria	1.6±0.3			
	Clostridia; Thermoanaerobacterales; Thermodesulfobiaceae; Coprothermobacter				3.9±2.2
Hydrogenedentes				0.6±0.9	
Latescibacteria		1.7±0.3		1.4±0.4	
Marinimicrobia	*SAR406 clade*		1.9±2.7		
Parcubacteria	*Candidatus Falkowbacteria*				0.7±1.5
Planctomycetes	*Planctomycetacia; Planctomycetales; Planctomycetaceae; Pir4 lineage*				0.3±0.5
	Planctomycetacia; Planctomycetales; Planctomycetaceae; Pirellula	1.6±1.6		1.2±0.3	
	Planctomycetacia; Planctomycetales; Planctomycetaceae;	0.5±0.9		2.3±0.3	1.1±0.7

Continues in the next page

		Feedstock scenario*			
		1	2	3	4
Proteobacteria	*Alphaproteobacteria; Rhizobiales; MNG7*			0.6±0.9	
	Alphaproteobacteria; Rickettsiales; Mitochondria			0.5±0.8	
	Betaproteobacteria; Rhodocyclales; Rhodocyclaceae; Thauera		1.6±0.5		
	Deltaproteobacteria; Syntrophorhabdaceae			0.5±0.7	
	Deltaproteobacteria; Syntrophobacterales; Syntrophaceae; Smithella;	**4.8±2.3**	**8.2±0.6**	**0.6±0.8**	**11.5±1.3**
Saccharibacteria			1.3±0.1		0.3±0.5
Spirochaetae	*Spirochaetes; Spirochaetales; Leptospiraceae;*			1.5±0.3	
	Spirochaetes; Spirochaetales; Spirochaetaceae;	2.5±1.9	2.4±0.1		
Synergistetes	***Synergistia; Synergistales; Synergistaceae***	**4.5±2.3**	**4.8±3.0**	**1.0±1.5**	**5.1±1.4**
Thermotogae	*Thermotogae; Kosmotogales; Kosmotogaceae; Mesotoga*			2.3±0.2	1.5±0.1
	Thermotogae; Petrotogales; Petrotogaceae; Defluviitoga				2.3±0.8
Verrucomicrobia	*L1-PB3*		1.5±0.2		
	OPB35 soil group	0.4±0.7			
	Spartobacteria; Chthoniobacterales; 12Z36				0.6±1.2
	Spartobacteria; Chthoniobacterales; Chthoniobacteraceae; Chthoniobacter	1.0±1.7			
	Verrucomicrobiae; Verrucomicrobiales; Verrucomicrobiaceae	2.1±0.6		1.8±0.4	0.3±0.6
	WCHB1-41	0.9±0.8		0.6±0.8	
WS6	*bacterium*	0.5±0.8			
WWE3				0.6±0.9	2.5±0.3

Relevant groups of the core microbiome have been highlighted in bold.

*Feedstock scenarios are: *Scenedesmus* (1), *Scenedesmus*+Primary Sludge (2), *Chlorella* (3), *Chlorella*+Primary Sludge (4).

**Only dominant *Archaea* phylum.

Table 7.6. Phyla level relative abundances of each sample related to each collection point to the AnMBR codigester.

	AnMBR co-digester								
Operation days	**0**	**96**	**103**	**188**	**205**	**363**	**482**	**419**	**440**
Euryarchaeota*	1.5	0.9	3.1	5.0	4.1	3.3	3.9	4.0	3.3
Acidobacteria	4.8	2.8	3.8	0.8	1.1	1.5	2.2	1.4	1.1
Actinobacteria	n.d.	1.2	1.0	1.7	2.3	1.9	1.8	2.5	2.4
Aminicenantes	0.9	0.6	0.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Atribacteria	0.5	0.6	0.9	1.4	1.1	2.1	2.6	2.2	2.0
Bacteroidetes	5.8	5.0	4.0	8.5	5.7	6.8	6.2	6.4	7.9
Caldiserica	1.0	1.3	0.9	n.d.	n.d.	4.0	5.8	7.5	7.5
Chloroflexi	23.3	25.9	25.3	27.6	29.1	14.5	16.3	18.9	17.6
Cloacimonetes	2.4	5.3	5.1	7.0	3.1	3.5	3.7	3.7	2.8
Firmicutes	10.5	7.3	7.3	3.7	4.2	11.2	9.6	7.1	6.3
Planctomycetes	4.1	3.8	5.4	2.2	2.9	4.1	3.6	2.4	3.4
Proteobacteria	18.3	21.9	17.7	22.6	23.7	22.5	21.9	21.9	17.9
Spirochaetae	13.0	10.8	10.7	2.4	2.7	0.7	0.8	1.0	1.1
Synergistetes	3.4	0.7	0.9	3.2	7.6	7.6	5.5	4.2	4.6
Thermotogae	n.d.	n.d.	n.d.	0.8	n.d.	3.2	3.8	4.5	5.2
Verrucomicrobia	4.4	6.8	7.0	3.5	4.7	3.4	3.5	3.9	5.1
WS6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
WWE3	n.d.	n.d.	n.d.	n.d.	n.d.	2.9	2.7	2.2	2.4

Non-detected phyla (n.d.)

*Only *Archaea* dominant phyla observed

Table 7.7. Phyla level relative abundances of each sample related to each collection point to the co-digester reservoir.

	Co-digester reservoir										
Operation days	**0**	**96**	**103**	**124**	**170**	**188**	**205**	**363**	**482**	**419**	**440**
Euryarchaeota*	1.7	2.1	2.7	2.8	3.8	3.5	6.3	4.0	4.9	4.6	4.2
Acidobacteria	3.3	2.3	5.4	5.5	1.3	1.4	1.0	2.9	2.6	2.0	1.8
Actinobacteria	1.0	0.6	1.9	2.0	2.2	2.3	1.6	2.2	2.6	2.6	2.4
Aminicenantes	0.7	n.d.	1.6	1.8	0.8	0.8	0.5	0.8	1.2	1.1	1.1
Atribacteria	0.6	n.d.	1.3	1.4	1.4	1.3	1.4	2.3	2.9	2.9	2.7
Bacteroidetes	3.1	10.8	2.8	3.0	5.0	4.9	7.1	5.9	5.1	5.7	6.2
Caldiserica	0.9	n.d.	0.7	0.7	0.7	0.6	n.d.	3.3	4.1	6.5	7.6
Chloroflexi	31.4	15.1	31.0	29.0	33.8	33.6	30.9	16.5	16.3	19.1	19.1
Cloacimonetes	4.2	6.5	4.0	4.0	8.7	8.5	7.4	3.7	3.4	4.3	3.9
Firmicutes	10.4	9.0	8.7	9.0	2.7	2.9	3.4	10.7	12.1	10.5	8.2
Planctomycetes	3.2	6.8	3.0	3.5	1.5	1.6	2.7	4.6	4.2	2.6	2.7
Proteobacteria	15.8	13.6	15.3	14.9	14.2	14.0	17.3	21.6	16.9	16.2	14.6
Spirochaetae	12.8	6.7	12.1	12.4	11.7	12.7	3.9	0.7	0.6	0.7	0.8
Synergistetes	1.1	9.7	0.9	1.0	1.3	1.2	3.6	7.9	9.6	6.9	7.5
Thermotogae	0.6	9.1	0.7	0.7	0.9	0.8	1.7	3.3	3.9	4.6	5.5
Verrucomicrobia	5.4	2.9	4.3	4.6	2.9	2.9	4.6	2.3	1.9	2.3	3.0
WS6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
WWE3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.2	1.9	2.1	2.0

Non-detected phyla (n.d.)

*Only *Archaea* dominant phyla observed

Table 7.8. Phyla level relative abundances of each sample related to each collection point to the AnMBR digester.

	AnMBR digester												
Operation days	**0**	**37**	**51**	**173**	**183**	**211**	**290**	**300**	**323**	**483**	**624**	**729**	**808**
Euryarchaeota*	2.4	3.6	1.3	1.4	1.5	0.7	2.7	2.5	1.2	2.1	1.5	2.9	2.9
Acidobacteria	0.0	0.6	0.5	2.9	3.0	2.6	4.4	2.9	2.3	0.9	0.0	1.1	0.0
Actinobacteria	2.0	1.4	0.6	1.3	2.1	1.6	1.1	1.4	0.9	0.0	0.9	2.7	2.1
Aminicenantes	0.0	0.0	0.0	0.0	1.2	0.0	9.0	8.2	4.5	4.2	1.3	4.6	2.8
Atribacteria	2.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.1	0.9
Bacteroidetes	4.7	2.7	4.2	3.8	4.0	6.5	12.1	10.5	19.4	9.6	5.6	4.9	5.3
Caldiserica	0.8	0.6	0.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.8	0.7	0.6
Chloroflexi	22.7	21.9	26.0	11.8	13.2	16.7	20.9	22.1	20.0	15.2	19.9	22.9	26.3
Cloacimonetes	2.3	3.5	12.3	2.0	2.1	5.0	1.0	1.2	3.4	5.9	8.0	7.4	4.6
Firmicutes	10.2	7.0	11.9	7.4	13.1	15.7	3.6	3.4	4.0	8.1	4.0	2.8	2.9
Planctomycetes	10.0	16.1	7.3	9.4	10.2	9.0	4.8	5.5	2.8	5.5	3.1	7.8	7.2
Proteobacteria	14.9	16.5	18.4	15.7	13.0	16.5	16.5	13.9	20.1	24.1	27.3	18.3	20.1
Spirochaetae	1.0	1.4	3.6	0.6	0.6	1.0	1.6	1.8	5.0	2.3	6.2	2.6	2.1
Synergistetes	13.0	8.2	2.9	5.8	3.9	3.8	4.8	6.8	2.2	7.0	2.4	1.4	2.9
Thermotogae	0.0	0.0	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.7	2.2	2.5
Verrucomicrobia	2.5	5.6	3.3	5.2	7.6	5.6	8.8	9.4	5.5	2.4	5.5	6.9	5.9
WS6	0.0	0.6	0.0	17.6	12.9	4.4	1.0	1.8	0.0	3.8	1.6	0.0	0.0
WWE3	6.8	4.9	1.6	7.6	2.2	1.2	0.0	0.0	0.0	0.0	0.0	1.3	0.6

Non-detected phyla (n.d.)

*Only *Archaea* dominant phyla observed

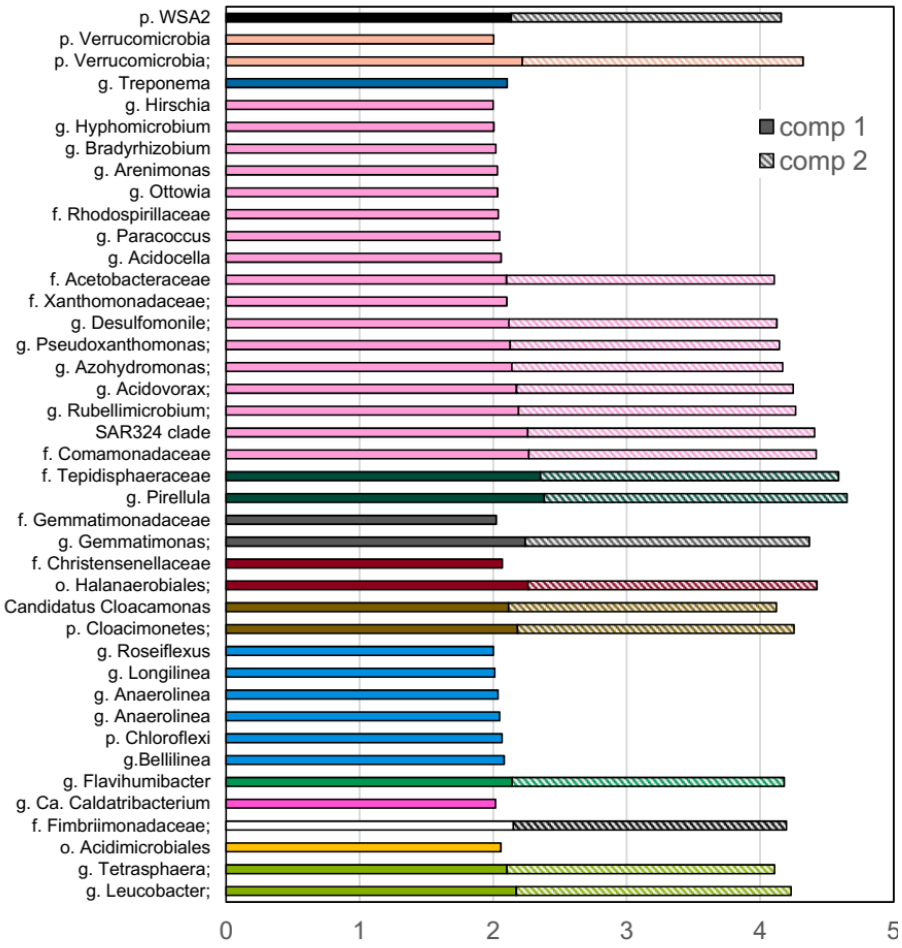

Figure 7.7. Variable Important Parameter (VIP) of the PLS-DA regression model for the first and second components. Threshold value set at 2.0 for the first and second components extracted. Groups belonging to the same phylum are shown in the same color.

8. Implementation of 16S rRNA/rDNA sequencing in layouts for resource recovery: a case study about microalgae co-digestion in an Anaerobic Membrane Bioreactor (AnMBR)

8. Implementation of 16S rRNA/rDNA sequencing in layouts for resource recovery: a case study about microalgae co-digestion in an Anaerobic Membrane Bioreactor (AnMBR)

Abstract

Microbial community composition has been widely studied through 16S rDNA sequencing. The diversity of different systems for microalgae digestion and co-digestion has been reported but lack discrimination of active members. In this study, the prokaryotic community of an anaerobic membrane bioreactor (AnMBR) plant converting raw microalgae and primary sludge into biogas has been evaluated through 16S rDNA and 16S rRNA sequencing to reveal both identity and activity sides of the microbial community. The effect of the microbial diversity of the feedstock over the AnMBR digestate has also been evaluated. The thermophilic AnMBR community showed lower diversity in terms of species richness than the mesophilic. The 16S rRNA community revealed higher fluctuation in terms of OTU distribution than the 16S rDNA. The feedstock associated microbial diversity had higher influence over mesophilic AnMBR active community (63% shared diversity), than over the thermophilic community (14%) since mesophilic members entering the thermophilic system within the feedstock did not thrive. During microalgae and primary sludge co-digestion, the active thermophilic community was dominated by *Coprothermobacter, Anaerobaculum, Fervidobacterium* and two methanogens, *Methanothermobacter* and *Methanosarcina*. The mesophilic performance resulted in an active community with a higher presence of *Anaerolineaceae*, *Mesotoga*, *Thermoanaerovibrio*, *Methanosaeta*, and *Methanoculleus*. These findings support the community composition reported in previous studies based on 16S rDNA sequencing but also discriminate high abundant non-active members such as *Comamonas* and *Mycobacterium* that were detected in high relative abundances in the AnMBR. Therefore, 16S rRNA/rDNA sequencing implementation in future AnMBR analysis could aid to elucidate the link between the performance associated information and the microbial community from the AnMBR after reduction of the background microbial diversity and discrimination of active members

Keywords

16S rRNA/rDNA; anaerobic membrane bioreactor (AnMBR); co-digestion; microalgae; microbial ecology

8.1.Introduction

The search for alternative and sustainable resources has become a need in a worldwide scenario of water, fuels, and nutrients scarcity. On this basis, used water has attracted the attention of the scientific community since it is considered a valuable source of energy, solids, reclaimed water and essential nutrients such as nitrogen and phosphorous (Verstraete *et al.,* 2016). Thus, the concept of Water Resource Recovery Facilities (WRRF) has replaced the conventional vision of wastewater treatment plants (WWTP) (Puyol *et al.,* 2017). Nowadays, innovative technologies are being combined to maximize resource recovery from wastewater (Seco *et al.,* 2018a).

Microbial biotechnology is essential to understand the biological processes for resource recovery since it is behind the organic matter degradation and the release of nutrients and high-value products and biofuels (Nielsen, 2017). As proposed by Batstone *et al.* (2015) platforms for circular economy from wastewater should be based on: (i) low strength anaerobic treatment and (ii) biological uptake-assimilation-accumulation of carbon and nutrients by phototrophic and/or heterotrophic cultures. Microalgae are an attractive alternative for resource recovery since they can be cultured over wastewater reducing the organic and nutrient loads (Acién *et al.,* 2016). Moreover, this phototrophic biomass can be transformed in biogas through anaerobic digestion processes (González-Fernández *et al.,* 2015), thus allowing a direct link between water treatment and bioenergy recovery.

During the last decade, several authors have explored bioenergy recovery from microalgae considering pre-treatment application (Passos *et al.,* 2014; Zamalloa *et al.,* 2012b, 2012a) and raw digestion conditions (González-Fernández *et al.,* 2018; Greses *et al.,* 2017; Klassen *et al.,* 2017; Sanz *et al.,* 2017; Zamorano-López *et al.,* 2019c, 2019a). Few studies have demonstrated the efficiency of microbial communities to acclimate to microalgae biomass and convert it into biogas in raw conditions using different reactor configurations (Greses *et al.,* 2018; Tartakovsky *et al.,* 2015; Zamorano-López *et al.,* 2019c). Greses *et al.,* (2017) reported that working at high retention times in Anaerobic Membrane Bioreactor

(AnMBR) promoted a hydrolytic community for raw microalgae digestion. The comprehension about identity, physiology, ecology and dynamics of relevant microbial groups could help to improve process stability (Nielsen, 2017), expanding anaerobic digestion microbial databases (McIlroy *et al.*, 2017) and allowing the design of strategies to anticipate to process failures (Carballa *et al.*, 2015). In this context, there is a growing interest on characterizing the microbial population of bioengineered systems for resource recovery when using bioengineered systems.

The abovementioned studies about microalgae digestion characterized the *Bacteria* and *Archaea* population behind microalgae digestion, contributing to the comprehension of their potential metabolic roles and the effect of bioreactor operational parameters. Klassen *et al.* (2017) observed a *Firmicutes* and *Thermotogae* dominance under stable nitrogen levels in the microalgae digester with the dominance of hydrogenotrophic methanogens. Similar findings were reported by Greses *et al.* (2017) during *Scenedesmus* anaerobic digestion who identified a novel *Thermotoga* group under thermophilic conditions. The study from Sanz *et al.* (2016) related the abundance of proteolytic *Firmicutes* members to the high protein content of *Chlorella* biomass during mesophilic digestion. Furthermore, the source of inoculum has been explored by Zamorano-López *et al.* (2019a), who observed a dominance of *Fervidobacterium* when using ruminal fluid for raw *Scenedesmus* degradation in an AnMBR. The election of the inoculum and its acclimation is an important strategy to guarantee a high methane yield achievement from raw microalgae (González-Fernández *et al.*, 2018). Moreover, some of these studies are very interesting from a commercial scale perspective since they have obtained the microalgae biomass from photobioreactor plants treating sewage (Solé-Bundó *et al.*, 2019; Zamorano-López *et al.*, 2019c).

Commercial systems for microalgae cultivation are commonly characterized by a complex phototrophic bacteria-eukaryotic community (Carney *et al.*, 2014). Hence, the harvested microalgae have an intrinsic associated microbial diversity. This aspect was explored by Greses *et al.*

(2017), who characterized the microbial composition of a *Scenedesmus* biomass harvested from a membrane photobioreactor plant (MPBR). The authors demonstrated that feedstock-associated diversity did not affect the thermophilic and mesophilic digestate communities. However, the influence of the continuous addition of other co-substrates (Li *et al.,* 2017; Zamorano-López *et al.,* 2019b) that also have an own microbial diversity (*e.g.* sewage sludge, rumen or manure) has not been reported in the literature. Also, no study using rDNA sequencing techniques has been complemented with the 16S rRNA gene characterization of systems for microalgae degradation to the knowledge of the authors. However, as pointed out by De Vrieze *et al.* (2018) the active communities provide a more accurate vision of stablished communities in anaerobic digesters. Since rRNA can discriminate between active and non-active microorganisms (De Vrieze *et al.,* 2016), the application of rRNA sequencing together with rDNA sequencing could improve the detection of active microbial communities.

This study moves toward the implementation of microbial molecular techniques in future technologies for resource recovery. The AnMBR plant used in this study represents the energy recovery step of a WRRF that applies anaerobic, phototrophic and membrane technologies to the treatment of urban wastewater (Seco *et al.,* 2018). This is the first study reporting the 16S rRNA/rDNA composition of digestate and rDNA feedstock-associated microbial communities of an AnMBR located in a WRRF. The changes in the diversity and structure of the bacterial and archaeal population are here evaluated for one year including thermophilic and mesophilic conditions. Hence, an innovative and upscaled vision of microbial communities for anaerobic digestion microalgae is here described. This study is a good example of how microbial approaches improve our understanding of complex biological processes for resource recovery such as biogas production from renewable sources like microalgae and primary sludge.

8.2.Materials and Methods

8.2.1. Operational conditions of the AnMBR plant

The AnMBR plant is located in the "Carraixet WWTP" (Valencia, Spain) and connected to the other two plants (see Figure 8.1), being part of an innovative layout for resource recovery from wastewater (Seco *et al.*, 2018). In this work, the AnMBR plant is referred to as a side-stream anaerobic membrane bioreactor ($AnMBR_{AD}$) to distinguish from the other AnMBR found in the WRRF. In the $AnMBR_{AD}$ takes place the co-digestion of the sludge stream resulting from primary settling, the biomass harvested from the MPBR, which is mainly composed of microalgae and the digestate from the AnMBR plant. But this last stream was not fed to the $AnMBR_{AD}$ in this study. Hence, the $AnMBR_{AD}$ worked as a co-digester for bioenergy recovery from microalgae and primary sludge.

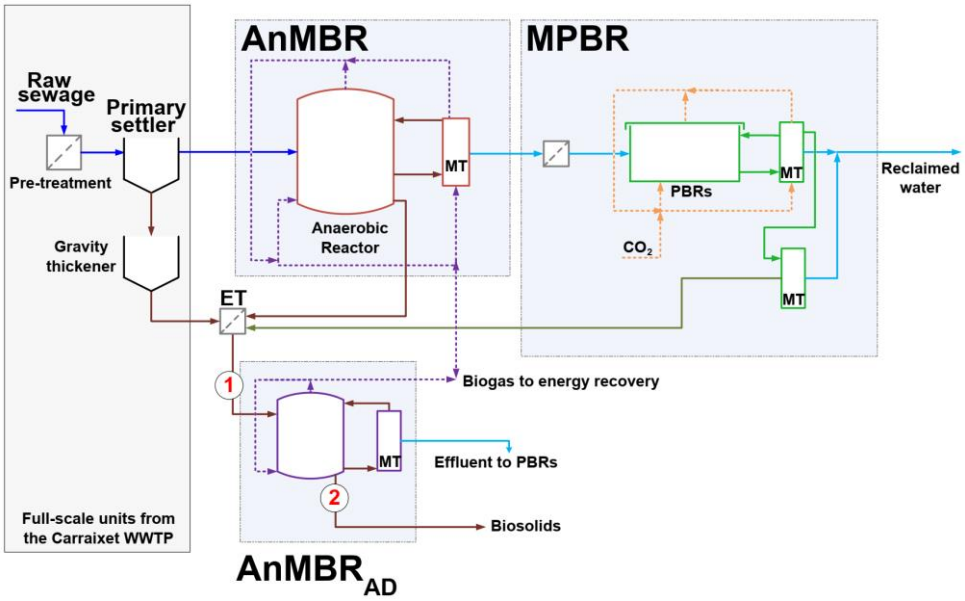

Figure 8.1. WRRF layout composed of an AnMBR plus a MPBR and the $AnMBR_{AD}$ used in this study. The numbers point the sample collection points used to collect feedstock biomass (point 1) and digestate (point 2). Abbreviations: Anaerobic Membrane Bioreactor (AnMBR), Equalization Tank (ET), Membrane Photobioreactor (MPBR), Membrane Tank (MT), Photobioreactor (PBR) and side-stream AnMBR ($AnMBR_{AD}$).

The $AnMBR_{AD}$ consists of a 1000 L main tank (maximum working volume was 900 L) coupled to a 1 L tank that contains an 0.42 m^2 ultrafiltration hollow-fibre unit (PURON® KMS, 0.03 µm pore size) (Koch Membrane Systems, USA). The different co-substrates fed to the tank are previously mixed in an equalization tank (ET) of 125 L. A heat resistance controls the temperature of the $AnMBR_{AD}$. A fraction of the biogas produced in the $AnMBR_{AD}$ is recycled to the bottom of the main tank. This mechanism favors the stripping of other gases released from the liquid phase during the anaerobic digestion process. Also, biogas assisted membrane scouring is applied to minimize the membrane cake layer in the $AnMBR_{AD}$ membrane tank.

8.2.2. Operating conditions, inoculum source, and biomass sample collection design for microbial ecology analysis

The $AnMBR_{AD}$ was operated for 382 days and it was subdivided into two periods: thermophilic (0-278 days) and mesophilic (278-382 days). The $AnMBR_{AD}$ solids retention time (SRT) was 70 days with decoupled hydraulic retention time (HRT) of 30 days for both thermophilic and mesophilic studies. These values were fixed according to previous experience in the laboratory scale (Greses, 2017) and using Design and Simulation of Activated Sludge Systems (DESASS) software (Ferrer *et al.*, 2008). The co-substrates fed to the plant were mixed in a mass proportion of 62% primary sludge and 38% microalgae (based on their volatile solid content). These values were obtained after simulation of the biomass production in the primary settler and in the MPBR for the WRRF influent.

Anaerobic sludge from the mesophilic municipal full-digester of "Carraixet WWTP" (Valencia, Spain) was used to inoculate the $AnMBR_{AD}$. This biomass was acclimated to thermophilic conditions at 55°C and only fed with primary sludge between 0-67 days. Microalgae and primary sludge co-digestion started after day 67 (Figure 2). The thermophilic pseudo steady-state period was reached between 200 and 274 days. The acclimated thermophilic biomass was partially used as an inoculum for the mesophilic

experimental period. The $AnMBR_{AD}$ was inoculated with a mix of anaerobic sludge from the mesophilic full-digester of "Carraixet WWTP" and the thermophilic digestate in a 1:1 volumetric ratio. Finally, the temperature of the $AnMBR_{AD}$ was decreased to 35°C to evaluate mesophilic co-digestion (278-382 days). Under mesophilic conditions, the pseudo steady-state conditions were achieved after 350 days.

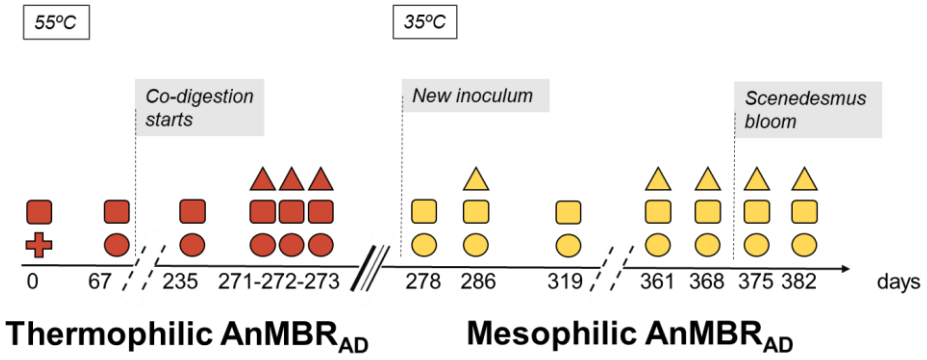

Figure 8.2. Schematic representation of the collection experimental design to characterize inoculum (cross), feedstock (squares), rDNA (circles) and rRNA (triangles) digestate communities.

Samples for microbial ecology analysis of the $AnMBR_{AD}$ plant were extracted from two different collection points (Figure 8.1). The first collection point was used to characterize the feedstock microbial community. The second collection point represents the digestate microbial community of the $AnMBR_{AD}$. The digestate samples were extracted in duplicate and separately stored for 16S rDNA (13 DNA digestate samples) or 16S rRNA (8 RNA digestate samples) analysis in 2 mL cryotubes. Feedstock samples were only stored for rDNA analysis using 2 mL cryotubes. The biomass was collected in parallel for both feedstock and digestate characterization of the $AnMBR_{AD}$. The biomass collection design is represented in Figure 8.2. Triplicated 700 µL aliquots from each feedstock or digestate sample were centrifuged at 5,000 x g for 3 minutes. The supernatant was removed, and the pellets were stored at -80ºC for rDNA analysis. For RNA analysis, the 2 mL cryotube was filled up with the nucleic

acid preservation reagent RNAlater® (Sigma-Aldrich, USA) to avoid the degradation of RNA and guarantee its stabilization over time.

8.2.3. Feedstock sources

The co-substrates were mixed in the ET before being fed to the $AnMBR_{AD}$ (Figure 8.1). The primary sludge co-substrate resulted from the primary settling and gravity thickening stages of the "Carraixet WWTP". The microalgae biomass was harvested using an external cross-flow, ultrafiltration hollow-fiber membrane unit (HF 5.0-43-PM500, PURON® Koch Membrane Systems, USA). The dominant species of microalgae were determined by the conditions of this MPBR plant and identified through microscopic observation. During this study *Scenedesmus* and *Chlorella* were the dominant genera of the MPBR. The performance of the MPBR and the other AnMBR located in the WRRF are extensively described in (González-Camejo *et al.*, 2019) and (Seco *et al.*, 2018), respectively.

8.2.4. Physicochemical analysis and biogas production

The different streams of the $AnMBR_{AD}$ were characterized according to standard methods (APHA, 2012) for the determination of solids and COD. Carbonate alkalinity and volatile fatty acids (VFA) were measured according to the instructions of the South African Water Research Commission (Moosbrugger *et al.*, 1993). These data were only used to identify the pseudo-steady state periods and proceed with the collection of representative biomass samples for microbial analysis. The pseudo-steady state $AnMBR_{AD}$ showed stability in terms of solids and COD concentration, and biogas production over time.

Biogas produced in the $AnMBR_{AD}$ was continuously measured using a BK-G4M gasometer (Manuel Romeu S.L., Spain). A 0.5 L sterilized airtight bag was used to collect the biogas samples before composition determination. The methane content was determined from 250 µL of biogas using a gas chromatograph fitted with a flame ionization detector (GC-FID, Agilent Technologies, USA). A gas-tight syringe was used for this purpose. The GC-FID was equipped with a TRACER column (Teknokroma) of 15 m

x 0.53 mm x 1 μm dimensions and set up at 40ºC. The flow rate of the carrier gas (Helium) was 5 $mL \cdot min^{-1}$. The quantification of the methane content in the sample was determined according to a high pure standard of methane gas (99.99% purity, Air Products, USA) calibration.

8.2.5. Nucleic acid extraction

The extraction of DNA was performed separately from RNA using different commercial kits. The DNA was extracted in duplicate within Qiagen Power Soil DNA kit (Qiagen, USA), following the procedures of the manufacturer with few modifications. Samples were centrifuged at 5000 x g for 3 minutes and the supernatant was discarded. The pellets were then suspended in the PowerBead solution (available in the Qiagen kit) and transferred to 2 mL tubes with 0.5 g of zirconium 0.1 mm diameter beads (Qiagen, USA) and C2 solution of the DNA kit. Bead-beating was performed using a BioSpec Bead Beater (BioSpec, USA). The tubes were then centrifuged at 10,000 x g for 1 minute and the supernatant was transferred to follow the Qiagen Power Soil DNA kit remaining instructions. The final DNA extracted was stored at -20ºC until high-throughput sequencing.

The extraction of RNA was performed in triplicate to retrieve a high amount of RNA per sample. The RNAlater® agent was removed from the samples before the extraction. The Maxwell 16 LEV Tissue RNA Kit (Promega, USA) was used for this purpose with similar modifications to those performed over biomass samples before DNA extraction. Thus, a bead-beating step with 0.5 g of zirconium 0.1 mm diameter beads was performed with addition of 300 μL of 1-Thioglycerol/Homogenization solution and 200 μL of lysis buffer (both reagents are provided in the RNA extraction kit). The disrupted solids were transferred to the kit cartridges and the rest of the procedure was performed according to the manufacturer in a Maxwell Machine (Promega, USA) equipped within a Maxwell 16® High Strength LEV Magnetic Rod and Plunger Bar Adaptor (Promega, USA). Negative controls from purified elution buffer were included in both DNA

and RNA extraction procedures. The negative control extractions were the last to be performed.

8.2.6. Quantification of purified nucleic acids

The different nucleic acid concentrations extracted from the different feedstock and digestate samples were quantified in duplicate in a Qubit 2.0 fluorometer (Invitrogen, USA) using both Qubit dsDNA and RNA HS assay kits (Invitrogen, USA) for DNA and RNA, respectively. Also, the $A_{260/280}$ and $A_{260/230}$ ratios of each sample were determined in duplicate with 1.5 µL of the sample using a spectrophotometer NanoDrop 2000 (Thermo Scientific, USA). These ratios referred to the quality of the nucleic acid extraction. High-quality DNA extraction commonly has an $A_{260/280}$ ratio of ~1.8 and ~2.0 for RNA. The $A_{260/230}$ are expected to be between 2.0-2.2 to guarantee the absence of co-extracted impurities such as humic acids and other phenolic compounds. Samples that met the abovementioned quality criteria were merged in a single aliquot corresponding to the feedstock and digestate collection points.

8.2.7. Synthesis of cDNA from RNA

The DNA co-extracted with RNA from the digestate samples was removed before the synthesis of cDNA using a Turbo DNA-free kit (Invitrogen, USA). For each sample, 10 µL of RNA were treated with 4 µL DNAase buffer, 4 µL rDNAase I Enzyme and 2 µL RNA-ase free sterilized water (all of the reagents are provided in the kit). The treatment was performed at a constant temperature of 37ºC for 30 minutes using an Eppendorf Mastercycler 5333 thermocycler (Eppendorf, Germany). An additional DNA-free treatment was applied to all samples with 4U rDNAase I Enzyme provided in the kit to ensure no amplification of co-extracted DNA. Synthesis of cDNA was finally performed according to the manufacturer instructions of the SuperScript™ VILO™ cDNA Synthesis Kit (Invitrogen, USA). The resulting cDNA samples were stored at -20ºC until high-throughput sequencing.

8.2.8. High-throughput sequencing

All DNA and cDNA samples were submitted for high-throughput sequencing of the 16S rRNA gene. The target gene was amplified using 515F (5'- GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') primers according to (Kozich *et al.*, 2013). The mock community ZymoBIOMICS Microbial Community DNA Standard (Zymo Research cat#D6306, USA) was included in the experiment during 16S rRNA library preparation. This sample contains different genomic DNA extracted from pure cultures of eight bacterial and two fungal strains and ca be used to determine errors in downstream analysis of the sequences. The 16S rRNA gene amplicons were sequenced using a MiSeq Illumina machine with v2 chemistry in a 2x250 paired-end run (Illumina, USA). The sequencing library preparation and sequencing were performed at the University of Michigan Host-Microbiome Initiative (HMI) (University of Michigan, USA).

8.2.9. Downstream sequencing analysis: diversity and biostatistics

The resulting raw sequences were downstream processed to remove chimeras following the *MiSeq_SOP* pipeline (website accession data was 23rd April 2019) using open-source *mothur* software (v.1.41.1). The resulting sequences were clustered in Operational Taxonomic Units at 3% dissimilarity threshold ($OTU_{0.97}$). The taxonomy was assigned according to the 11.5 release of the Ribosomal Database Project (RDP) database.

The $OTU_{0.97}$ contingency table was used as an input for microbial ecology analysis using R-studio v.3.2 software and *vegan* packages. The relative presence of the $OTU_{0.97}$ in rDNA samples was used to calculate relative abundance. The relative activities were determined from the $OTU_{0.97}$ counts in RNA samples. The alpha diversity estimators provided by *mothur* were used to determine the species richness and the evenness of each feedstock, rDNA or rRNA digestate sample. This estimation was performed after rarefaction to 9905 sequences, which was the lowest sequencing size retrieved from the sequencing approach.

A non-metric multidimensional analysis (NMDS) using the Bray-Curtis distance matrix retrieved from *mothur* was used to explore the beta diversity of the $AnMBR_{AD}$. The analysis of similarities (ANOSIM) test was used to test the null hypothesis that the similarity between the different types of samples is higher than or equal to the similarity within them. Two tests combining NMDS and ANOSIM analysis were separately performed. The first evaluated the beta diversity and similarity of three types of samples collected from the plant: rDNA digestate, rRNA digestate and feedstock. The second test was performed over digestate rDNA and rRNA samples. Finally, Venn diagrams were constructed using the online web tool from (Bardou *et al.,* 2014) for thermophilic and mesophilic conditions. Only samples that were taken under pseudo-steady state conditions were used as biological replicates for Venn diagram analysis.

8.3.Results and Discussion

8.3.1. $AnMBR_{AD}$ performance

The main parameters extracted from the $AnMBR_{AD}$ operation and performance are summarized in Table 1. The harvested microalgae from the MPBR were mainly composed of *Chlorella,* but during the mesophilic operation a bloom of *Scenedesmus* spp. was observed as reported by González-Camejo *et al.* (2019). However, the changes in the species of microalgae did not have an effect on the $AnMBR_{AD}$ performance in terms of biogas production, which remained stable. The methane yield during the thermophilic period was 140 $mLCH_4 \cdot gCOD_{inf}^{-1}$, which was lower than the yield observed during mesophilic operation (205 $mLCH_4 \cdot gCOD_{inf}^{-1}$). This could be attributed to partial inhibition of the methanogenic population during the thermophilic performance. Indeed, accumulation of 455 $mgCH_3COOH \cdot L^{-1}$ was observed during pseudo-steady state thermophilic conditions while only 24 $mgCH_3COOH \cdot L^{-1}$ were determined during mesophilic operation.

Table 8.1. Main $AnMBR_{AD}$ plant performance and operational data including mean and standard deviation values retrieved from the pseudo-steady state conditions reached.

	Thermophilic	**Mesophilic**
Temperature (°C)	55.5 ± 1.5	35.2 ± 0.5
Microalgae Feedstock	*Chlorella*	*Chlorella, Scenedesmus*
SRT (d)	70.3 ± 5.5	70.2 ± 4.6
HRT (d)	30.7 ± 2.4	30.1 ± 4.4
pH	7.3 ± 0.3	7.2 ± 0.1
Methane yield ($mLCH_4 \cdot gCOD_{inf}^{-1}$)	140 ± 29	205 ± 5
Methane biogas content (%)	61.5 ± 6.6	67.1 ± 5.8
Volatile fatty acids (mg $CH_3COOH \cdot L^{-1}$)	455 ± 100	24 ± 33
Alkalinity (mg $CaCO_3 \cdot L^{-1}$)	1780 ± 50	2130 ±37

8.3.2. The effect of choosing 16S rDNA or rRNA sequencing over alpha diversity

The alpha diversity of $AnMBR_{AD}$ samples was evaluated using the observed species richness indicator (Figure 8.3a) and the inversed Simpson evenness index (Figure 8.3b). The highest diversity was observed in the feedstock samples. The lowest diversity was related to the rRNA samples analyzed in the thermophilic operation of the $AnMBR_{AD}$. In contrast, the analysis of mesophilic rRNA samples revealed a high species richness which was similar to the feedstock diversity. This could be related to the higher content of RNA than DNA in the cells (Jeffrey *et al.*, 1996) and the consequent higher sensitivity of rRNA-based sequencing, compared to rDNA. Microalgae feedstock harvested from the same MPBR in a previous study (Greses *et al.*, 2017) had higher diversity: more than 2000 $OTU_{0.97}$ were detected while in the mesophilic and thermophilic digestate samples accounted for 1396 $OTU_{0.97}$ and 977, respectively. In our study, the thermophilic rRNA, rDNA and the mesophilic rDNA samples also had a lower diversity than feedstock. However, our results partially disagree with the study from Greses *et al.* (2017) since mesophilic rRNA samples and feedstock had a similar number of $OTU_{0.97}$ (899±27 and 1123±133 mean values, correspondingly). Feedstock samples contain a fraction of the species that could also be active in the digestate samples. Besides, more microorganisms were detected from rRNA than from rDNA in mesophilic $AnMBR_{AD}$ samples, suggesting a higher sensitivity of rRNA sequencing. In

contrast, this was not observed in the thermophilic conditions since the high temperature applied to the system excludes non-thermophilic microorganisms and decreases species richness avoiding the "background" effect of low abundant microorganisms that could be more easily detected through rRNA than rDNA.

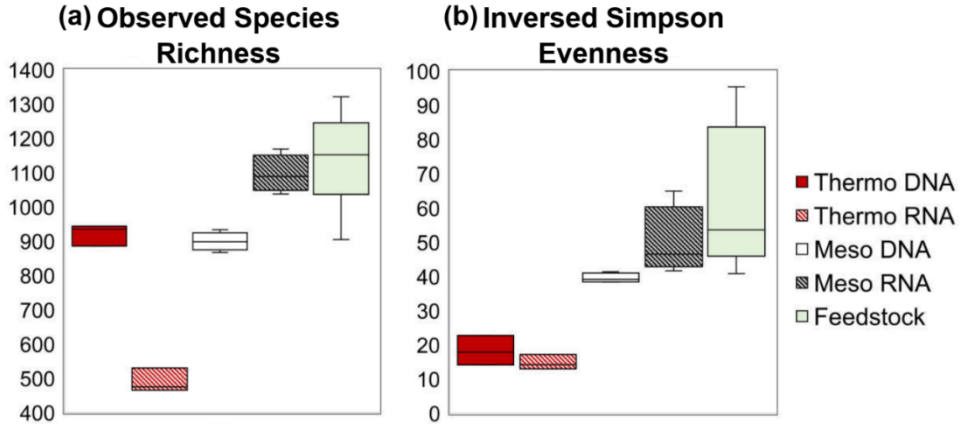

Figure 8.3. Alpha diversity measurements among the different samples collected from the $AnMBR_{AD}$: species observed richness (a) and inversed Simpson evenness (b) indexes.

Finally, the measurement of the evenness of the samples (Figure 8.3b) shows that the thermophilic community in the $AnMBR_{AD}$ was less diverse than the mesophilic. Again, this is related to the selective pressure generated at high temperatures of 55ºC compared to 35ºC operation. Furthermore, the thermophilic rRNA community had a lower diversity in terms of evenness than the rDNA since rRNA discriminates between active and non-active microorganisms. On the contrary, the mesophilic rRNA community was more diverse than the rDNA (Figure 8.3b). This should be attributed as for the observed species richness, to the higher sensitivity of rRNA sequencing. It should be highlighted that some authors have pointed out the complexity of correlating the levels of RNA in cells with activity since different life strategies such as dormancy disturb the RNA content of the cell (Emerson *et al.*, 2017). As suggested by Blazewicz *et al.* (2013), the interpretation of the rRNA digestate community should be carefully carried out.

8.3.3. Feedstock microbial diversity and temperature shaped the $AnMBR_{AD}$ microbial structures

The microbial structures observed among feedstock and digestate samples taken from the $AnMBR_{AD}$ plant are shown in the NMDS plot (Figure 8.4a). The ANOSIM test confirmed that the differences between feedstock, rDNA and rRNA communities shown in Figure 8.3a are significant (R_{ANOSIM}=0.575, $p<0.001$). Also, the same test performed over the digestate communities shown in Figure 8.4b determined that both rRNA and rDNA structures were significantly different (R_{ANOSIM}=0.519, $p<0.001$).

According to the NMDS analysis, the microbial diversity of the $AnMBR_{AD}$ digestate shifted after 67 days of acclimation to the thermophilic conditions (Figure 8.4a, 8.4b). Another change in this microbial structure was observed after 235 days of operation. Microalgae composition is complex and works as a selective pressure parameter over microbial population due to its content in hemicelluloses, celluloses or *algaenan* (Baudelet *et al.*, 2017). Hence, a specialization of the community is observed over time due to the temperature and feedstock composition. The community structure observed after 235 days in this study represents the microbial profile during thermophilic co-digestion of primary sludge and microalgae since the $AnMBR_{AD}$ reached pseudo-steady state conditions after 211 days. Different operational problems took place in the $AnMBR_{AD}$ after 235 days, inducing changes in the partial pressure equilibrium and apparently modifying the microbial structures (performance data not shown). Some microorganisms are very sensitive to these changes, such as fermentative bacteria and methanogens whose metabolism depends on hydrogen partial pressure (Venkiteshwaran *et al.*, 2015). This would explain the changes in both rDNA and rRNA communities observed after 235 days in the thermophilic $AnMBR_{AD}$ digestate samples (Figure 8.3b).

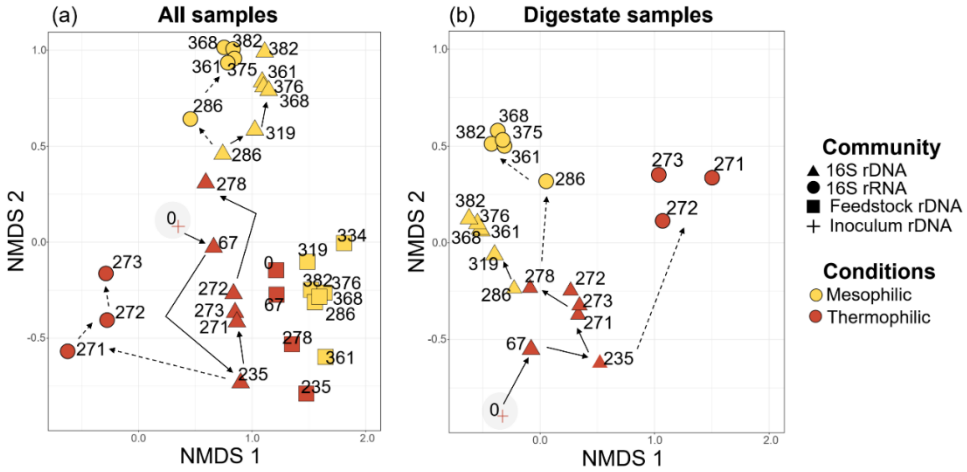

Figure 8.4. Non-metric multidimensional analysis (NMDS) of all (a) or only digestate (b) $AnMBR_{AD}$ samples. Text labels show the operational days during thermophilic (red color) and mesophilic (yellow color) performance. Shape differenciates between inoculum (crosses), feedstock (squares), digestate rDNA (triangles) and rRNA (circles). The NMDS was performed over the Bray-Curtis distance matrix for all samples (8.4a, stress value of 0.1481) and without feedstock samples (8.4b, stress value of 0.1409). Arrows have been added to each NMDS plot to facilitate chronological changes interpretation through DNA (solid) and RNA (dashed) analysis.

The temperature of the $AnMBR_{AD}$ was decreased to evaluate mesophilic co-digestion after 278 days of operation under thermophilic conditions. A different microbial structure resulted from mixing thermophilic digestate with fresh mesophilic digestate from the full-scale anaerobic digester of the municipal WWTP (see sample 278, Figure 8.4b). A specialization trend can be observed among mesophilic digestate samples, suggesting that mesophilic conditions and feedstock characteristics shaped the microbial structure of the new inoculum. On the contrary to thermophilic operation, the mesophilic population was more stable over time in terms of microbial diversity. This was elucidated from the short distance between 286-382 days rDNA digestate samples and also from the short distance between equivalent RNA samples. Moreover, no changes were observed in the $AnMBR_{AD}$ digestate community despite the *Scenedesmus* bloom in the MPBR (Figure 8.2). These findings highlight the robustness of the mesophilic community. This is in agreement with previous results using mesophilic lab-scale

AnMBRs fed with the similar primary sludge and microalgae biomass (Zamorano-López *et al.*, 2019b).

A certain distance between rDNA and rRNA communities was observed regardless of the temperature set in the $AnMBR_{AD}$. This distance is shown more clearly in Figure 8.4b due to the exclusion of feedstock samples which were only characterized using rDNA. This was also reported in the study about active communities in anaerobic digesters by De Vrieze *et al.* (2018). Also, Joyce *et al.* (2018) observed that the rRNA community structure was different from the rDNA according to NMDS analysis. Hence, it can be concluded that rDNA and rRNA sequencing reveal similar microbial communities with different species distributions for the same bioreactor conditions. This could be related, among other factors, to the establishment of different gradients of substrates (Ziels *et al.*, 2017), nutrients (De Vrieze *et al.*, 2016) or temperature (Lin *et al.*, 2016) in the $AnMBR_{AD}$ and also to changes in the inter-species relations (Narihiro *et al.*, 2018). The long-distance observed between digestate rDNA and rRNA from feedstock samples collected during mesophilic operation suggests the high acclimation level reached for microalgae and primary sludge co-digestion in the $AnMBR_{AD}$. In contrast, the rDNA thermophilic samples were more similar to the feedstock samples but the rRNA thermophilic were further apart. This could be attributed to the higher capacity of rRNA sequencing to discriminate between accumulated non-active microorganisms from the active groups.

8.3.4. Evaluating the effect of the microbial diversity detected in the feedstock over the $AnMBR_{AD}$ active community

In our study, the cells can be more easily detected using rRNA than rDNA as long as they survive in active or latent forms. As previously discussed, this would only apply for mesophilic conditions in our study since the temperature applied to the thermophilic $AnMBR_{AD}$ (55ºC) reduced the background microbial population. The microorganisms that entered the system within the feedstock did not tolerate this high temperature and their abundances decayed over time due to physical damage, disruption, cell death

or predation carried out by other groups. As it is shown in a Venn diagram form in Figure 8.5a, feedstock and active thermophilic rRNA community only shared the 14.1% of the diversity. On the contrary, rRNA mesophilic community shared a 62.9% diversity with feedstock samples (Figure 8.5b). This supports the concept of a background community related to the feedstock that remains in high retention systems such as the mesophilic $AnMBR_{AD}$ which was operated at a high SRT of 70 days.

Regarding the shared $OTU_{0.97}$ between feedstock and rDNA samples, the percentage was 50.1% and 69.5% for thermophilic and mesophilic, respectively. This agrees with the previous discussion about the detection of cells that are accumulated in the $AnMBR_{AD}$ but might not be active. Hence, the detection of rRNA might be a better approach to characterize microbial communities in high retention solid systems such as the $AnMBR_{AD}$ fed with microbial-rich feedstocks, but only when the system works at a different temperature range than the system used to retrieve the feedstock (in this case, a primary settler and MPBR plant).

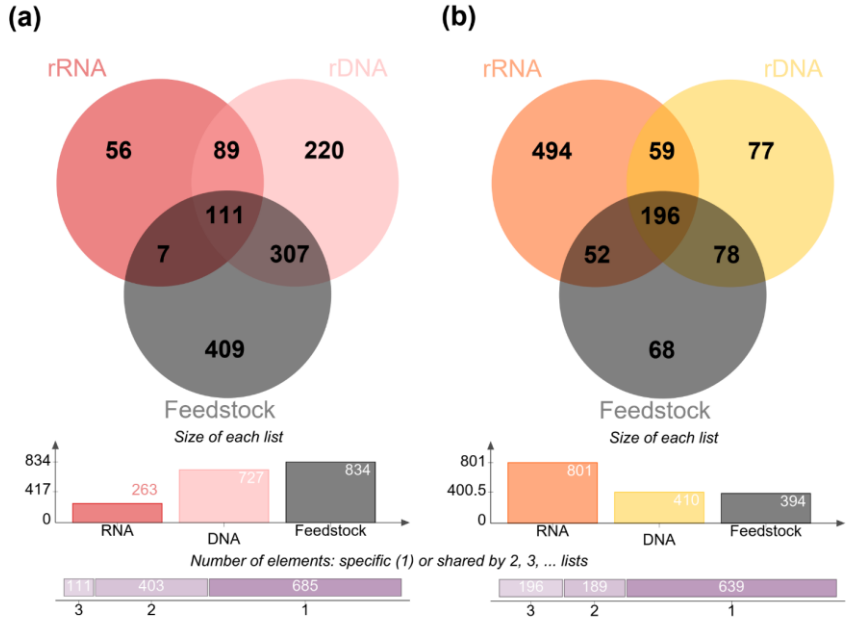

Figure 8.5. Venn diagrams from the microbial diversity observed in $AnMBR_{AD}$ (a) thermophilic and (b) mesophilic samples.

Table 8.2. Feedstock dominant composition in terms of relative abundance mean and standard deviation in the $AnMBR_{AD}$.

$OTU_{0.97}$	Bacteria	%rDNA
#003	*Proteobacteria;Betaproteobacteria;Burkholderiales; Comamonadaceae;Comamonas*	2.1±1.2
#005	*Proteobacteria;Epsilonproteobacteria;Campylobacterales; Campylobacteraceae; Arcobacter*	2.9±3.1
#009	*Bacteroidetes;Bacteroidia;Bacteroidales;Bacteroidaceae; Bacteroides*	4.3±1.6
#013	*Bacteroidetes;unclassified*	3.9±1.8
#014	*Unclassified*	0.1±0.1
#016	*Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae; Parabacteroides*	2.6±1.1
#026	*Proteobacteria;Betaproteobacteria;Rhodocyclales; Rhodocyclaceae;Thauera*	0.3±0.3
#034	*Firmicutes;Clostridia;Clostridiales;Peptostreptococcaceae; Clostridium XI*	0.1±0.0
#038	*Proteobacteria;Gammaproteobacteria;Pseudomonadales; Moraxellaceae; Acinetobacter*	0.3±0.3
#062	*Proteobacteria;Gammaproteobacteria;Pseudomonadales; Pseudomonadaceae;unclassified*	0.2±0.2
#092	*Proteobacteria;Alphaproteobacteria;unclassified*	0.1±0.2

*The percentage shows the relative abundance mean and standard deviation of the $OTU_{0.97}$ present in all samples colected between days 235 and 415 in the $AnMBR_{AD}$.

8.3.5. Characterization of the $AnMBR_{AD}$ communities

a. Feedstock community

The composition of the feedstock shown in Table 8.2 highlights the dominance of *Proteobacteria* and *Bacteroidetes* members. Most of these $OTU_{0.97}$ showed lower values of abundance or activity in the $AnMBR_{AD}$ digestate (marked in Tables 8.3 and 8.4), compared to the feedstock (Table 8.2). Only $OTU_{0.97}$ #003 *Comamonas* was observed in higher abundances in thermophilic digestate rDNA samples (4.8%), compared to the rest of the feedstock or digestate samples. The higher values of abundance observed for this bacteria are in accordance with the findings of Sanz *et al.* (2017) who pointed out the survival of these bacteria as resting forms in anaerobic digesters. However, the relative activity of *Comamonas* decayed over time under thermophilic conditions, as well as its relative abundance (Figure 8.4). Moreover, it was only detected during the start-up of mesophilic

experimental phase. Similarly, most of the $OTU_{0.97}$ found in the feedstock samples that were also observed in the digestate had a negative relative abundance and activity trend in the $AnMBR_{AD}$ digestate (Tables 8.3 and 8.4). The feedstock diversity observed in this study is similar to the results reported by Greses *et al.* (2017) who characterized the microalgae harvested from the MPBR plant in a previous experiment. The authors determined a dominance of 38.6% *Proteobacteria* and 15.8% *Firmicutes* and 15.3% *Bacteroidetes* among other phyla below 10.0%. In our study a higher content of 32.2% *Bacteroidetes* with 28.4% *Proteobacteria* and much lower abundance of *Firmicutes* (9.8%) were observed. These changes would be attributed to the diversity of primary sludge and fluctuations in the 16S rDNA community of the MPBR plant.

a. $AnMBR_{AD}$ Digestate community

The microbial profile of the $AnMBR_{AD}$ digestate revealed important changes in terms of composition and $OTU_{0.97}$ relative abundance over time (Figure 8.4). The analysis from rDNA and rRNA of the acclimation and later pseudo-steady state stages of the thermophilic $AnMBR_{AD}$ highlights the importance of several bacteria phyla such as *Firmicutes* ($OTU_{0.97}$ #001, #020, #029, #034), *Planctomycetes* ($OTU_{0.97}$ #030), *Synergistetes* ($OTU_{0.97}$ #007) and *Thermotoga* ($OTU_{0.97}$ #004, #015). Also, different methanogens belonging to the *Archaea Euryarchaeota* phylum were observed ($OTU_{0.97}$ #011, #012, #017 and #025). The acclimated biomass established during thermophilic performance of the $AnMBR_{AD}$ was mixed with fresh mesophilic sludge and used as a new inoculum for the mesophilic study. The use of previously acclimated biomass might help during the start-up of new operational conditions since long times are needed to achieve good acclimation levels for raw microalgae digestion.

Table 8.3. Relative abundance and activities in terms of mean and standard deviation of dominant $OTU_{0.97}$ detected in the thermophilic $AnMBR_{AD}$.

$OTU_{0.97}$	*Archaea*	%rDNA	%rRNA
#011	*Euryarchaeota;MethanoMethanobacteriales;Methanobacteriaceae;Methanothermobacter*	1.3±0.2	13.9±0.7
#012	*Euryarchaeota;Methanomicrobia;Methanosarcinales;Methanosaetaceae;Methanosaeta*	0.2±0.1	0.3±0.4
#017	*Euryarchaeota;Methanomicrobia;Methanosarcinales;Methanosarcinaceae;Methanosarcina;*	5.1±0.4	4.8±0.7
#025	*Euryarchaeota;Methanomicrobia;Methanomicrobiales;Methanomicrobiaceae;Methanoculleus;*	0.2±0.1	0.7±0.5
$OTU_{0.97}$	***Bacteria***		
#001	*Firmicutes;Clostridia;Thermoanaerobacterales;Thermodesulfobiaceae;Coprothermobacter*	20.3±3.1	17.0±2.4
#003*	*Proteobacteria;BetaProteobacteria;Burkholderiales;Comamonadaceae;Comamonas*	4.8±0.2	0.7±0.4
#004	*Thermotogae;Thermotogae;Thermotogales;unclassified*	5.0±0.4	1.9±0.5
#005*	*Proteobacteria;EpsilonProteobacteria;Campylobacterales;Campylobacteraceae;Arcobacter*	1.3±0.2	0.7±0.3
#007	*Synergistetes;Synergistia;Synergistales;Synergistaceae;Anaerobaculum*	2.2±0.3	9.8±0.6
#008	*Unclassified*	0.6±0.1	0.4±0.0
#009*	*Bacteroidetes;Bacteroidia;Bacteroidales;Bacteroidaceae;Bacteroides*	0.9±0.1	n.d.
#013*	*Bacteroidetes;unclassified*	0.9±0.1	n.d.
#014*	*Unclassified*	0.5±0.4	0.2±0.1
#015	*Thermotogae;Thermotogae;Thermotogales;Thermotogaceae;Fervidobacterium*	5.7±0.5	1.9±0.3
#016*	*Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae;Parabacteroides*	1.3±0.0	n.d.
#020	*Firmicutes;unclassified*	1.3±0.1	3.7±0.3
#024	*Unclassified*	2.3±0.2	1.5±0.1
#026*	*Proteobacteria;BetaProteobacteria;Rhodocyclales;Rhodocyclaceae;Thauera*	1.6±0.2	0.9±0.3
#029	*Firmicutes;Clostridia;Clostridiales;Syntrophomonadaceae;Syntrophomonas*	3.5±0.2	1.3±0.5
#030	*Unclassified*	0.8±0.0	4.6±0.6
#034*	*Firmicutes;Clostridia;Clostridiales;Peptostreptococcaceae;Clostridium_XI*	0.2±0.1	2.2±0.3
#038*	*Proteobacteria;GammaProteobacteria;Pseudomonadales;Moraxellaceae;Acinetobacter*	0.1±0.1	2.2±2.2

In total n=3 rDNA and rRNA digestate samples were used as biological replicates of the thermophilic co-digestion in the $AnMBR_{AD}$
*Indicates which $OTU_{0.97}$ were also found in the feedstock samples. BLASTed unclassified groups (%identity score): #004 *Defuviitoga tunisiensis* (100%), #006 *Mesotoga prima* (100%), #008 *Thermoanaerobacter* (89%), #013 *Sphingobacterium* (83%) , #014 *Mycobacterium* (80%), #020 *Calditerricola* (89%), #024 unknown and #030 *Thermogutta terrifontis* (97%). The percentage indicates the identity score retrieved after BLAST.

Table 8.4. Relative abundance and activities in terms of mean and standard deviation of dominant $OTU_{0.97}$ detected in the mesophilic $AnMBR_{AD}$.

$OTU_{0.97}$	**Archaea**	**%rDNA**	**%rRNA**
#012	Euryarchaeota;Methanomicrobia;Methanosarcinales;Methanosaetaceae;Methanosaeta;	2.4±0.4	5.8±0.8
#025	Euryarchaeota;Methanomicrobia;Methanomicrobiales;Methanomicrobiaceae;Methanoculleus;	0.3±0.1	3.5±1.1
$OTU_{0.97}$	**Bacteria**		
#001	Firmicutes;Clostridia;Thermoanaerobacterales;Thermodesulfobiaceae;Coprothermobacter	5.0±2.0	1.5±0.6
#002	Chloroflexi;Anaerolineae;Anaerolineales;Anaerolineaceae;unclassified	7.8±1.7	6.2±0.6
#003*	Proteobacteria;BetaProteobacteria;Burkholderiales;Comamonadaceae;Comamonas	1.0±0.4	0.5±0.2
#005*	Proteobacteria;EpsilonProteobacteria;Campylobacterales;Campylobacteraceae;Arcobacter	0.3±0.1	1.3±0.3
#006	Thermotogae;Thermotogae;Thermotogales;Thermotogaceae;unclassified	8.1±3.0	4.0±1.0
#008	Unclassified	3.8±1.0	0.3±0.1
#010	Bacteroidetes;unclassified	5.6±0.9	0.5±0.1
#014*	Unclassified	1.5±0.5	0.3±0.1
#015	Thermotogae;Thermotogae;Thermotogales;Thermotogaceae;Fervidobacterium	0.8±0.3	0.3±0.2
#016*	Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae;Parabacteroides	0.4±0.1	0.1±0.0
#020	Firmicutes;unclassified	0.6±0.2	1.0±0.2
#021	Unclasified	0.3±0.1	7.0±2.5
#023	Unclassified	2.0±0.5	1.5±0.7
#026*	Proteobacteria;BetaProteobacteria;Rhodocyclales;Rhodocyclaceae;Thauera	0.4±0.2	0.7±0.2
#034*	Firmicutes;Clostridia;Clostridiales;Peptostreptococcaceae;Clostridium_XI	0.1±0.0	1.4±0.4
#035	Bacteroidetes;unclassified	1.9±0.1	0.3±0.1

In total n=4 rDNA and rRNA digestate samples were used as biological replicates of the mesophilic co-digestion in the $AnMBR_{AD}$. *Indicates which $OTU_{0.97}$ were also found in the feedstock samples. BLASTed unclassified groups (%identity score): #002 *Leptolinea* (91%), #006 *Mesotoga prima* (100%), #008 *Thermoanaerobacter* (89%), #010 *Saccharicrinis* (86%), #014 *Mycobacterium* (80%), #020 *Calditerricola* (89%), #021 *Thermanaerovibrio* (84%) , #023 *Flexilinea* (95%) and #035 *Labilibacter* (90%).

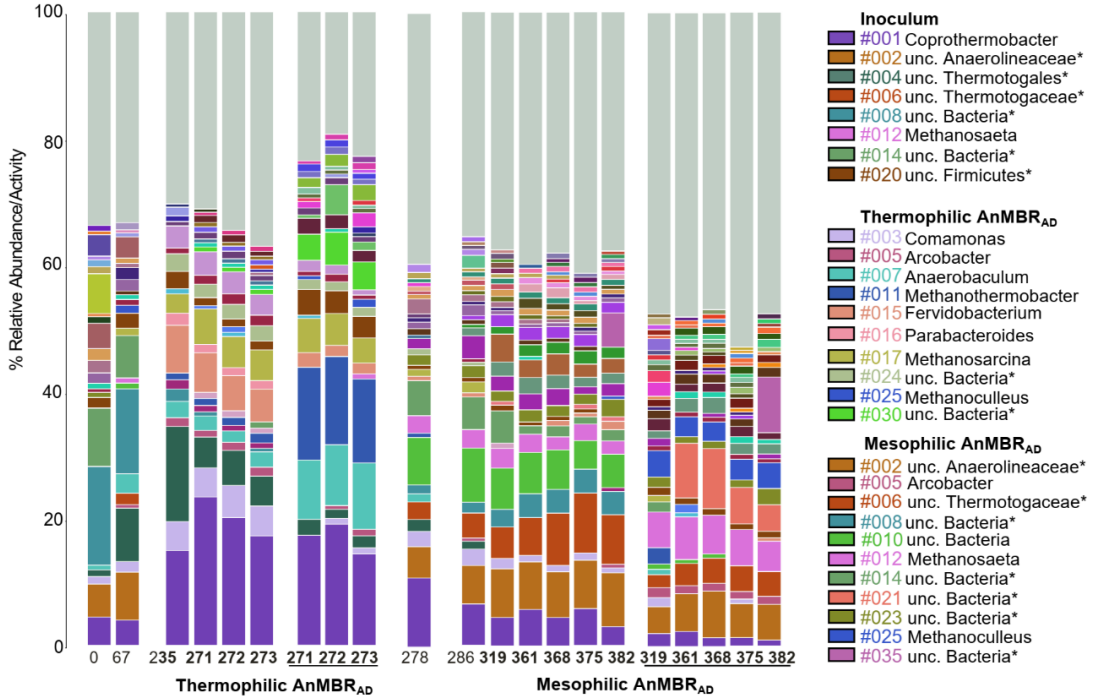

Figure 8.6. AnMBR$_{AD}$ digestate relative abundance of OTU$_{0.97}$ over 2.0% (groups below this value are summarized in grey barplot). *BLASTed unclassified groups: #002 *Leptolinea* (91%), %), #004 *Defuviitoga tunisiensis* (100%), #006 *Mesotoga prima* (100%), #008 *Thermoanaerobacter* (89%), #014 *Mycobacterium* (80%), #020 *Calditerricola* (89%), #021 *Thermanaerovibrio* (84%) , #023 *Flexilinea* (95%), #024 unknown, #030 *Thermogutta terrifontis* (97%) and #035 *Labilibacter* (90%). The percentage indicates the identity score retrieved after BLAST comparison.

The composition of the inoculum of the mesophilic $AnMBR_{AD}$ performance had high abundance of several $OTU_{0.97}$ from the previous thermophilic operation such as *Anaerobaculum* ($OTU_{0.97}$ #007), *Coprothermobacter* ($OTU_{0.97}$ #001), *Defluviitoga* ($OTU_{0.97}$ #004) or *Methanothermobacter* ($OTU_{0.97}$ #011). However, the mesophilic temperature enhanced other groups from *Chloroflexi* ($OTU_{0.97}$ #002 and #023), *Proteobacteria* ($OTU_{0.97}$ #005 and #020), *Synergistetes* ($OTU_{0.97}$ #021), *Thermotoga* ($OTU_{0.97}$ #006) Bacteria phyla and *Euryarchaeota* ($OTU_{0.97}$ #012 and #025) *Archaea* phylum. These $OTU_{0.97}$ were more promoted than the others that had remained presumably active after the mesophilic start-up. Hence, they dominated the microbial population of the mesophilic $AnMBR_{AD}$ after 361 days of operation, as shown in Figure 8.4. Most of the groups observed in the $AnMBR_{AD}$ under thermophilic and mesophilic conditions have been previously found in different studies about anaerobic digestion of raw microalgae (González-Fernández *et al.,* 2018; Greses *et al.,* 2017; Klassen *et al.,* 2017; Sanz *et al.,* 2017; Zamorano-López *et al.,* 2019c, 2019a) and in co-digestion (Li *et al.,* 2017b; Zamorano-López *et al.,* 2019b). Up to date and to the knowledge of the authors, no prior study about biogas production from microalgae has applied other omics-approaches to confirm the suggested metabolic roles of these microorganisms. This is the first study revealing their relative activities values in continuous systems using rRNA sequencing of the 16S rDNA.

A global trend during both thermophilic (Table 8.3) and mesophilic (Table 8.4) periods was that the relative activities of archaea were higher than their relative abundances. The opposite was observed for bacteria. This could be related to the discrimination of non-active groups that decreased the diversity of thermophilic samples (Figure 8.3a, 8.3b). For mesophilic samples, this would only apply for dominant $OTU_{0.97}$ since the alpha diversity analysis of mesophilic samples revealed that the total rRNA community have a higher richness and evenness than the rDNA (Figure 8.3a, 8.3b). Lower values of alpha diversity were also observed for archaea compared to bacteria by De Vrieze *et al.* (2018). The study evaluated the

microbial community of different thermophilic and mesophilic full-scale digesters through 16S rRNA/rDNA amplicon sequencing. Overall, some aspects that are not evaluated in our study could modify the $OTU_{0.97}$ distribution in the $AnMBR_{AD}$: (i) inter-species relationships (Ziels *et al.*, 2018), (ii) potential process inhibitors (De Vrieze *et al.,* 2015; Lin *et al.*, 2017) and (iii) spatial variation due to membrane film development (Smith *et al.,* 2015). These aspects should be considered in future studies that search the correlation between operational parameters and the representative microbial composition of microalgae co-digestion.

b. Thermophilic co-digestion

The diversity observed in the thermophilic $AnMBR_{AD}$ suggests the implication of three dominant $OTU_{0.97}$ identified as *Coprothermobacter* (#001), *Fervidobacterium* (#015) and *Thermogutta* (#030) in the hydrolysis and later fermentation of the polysaccharide and protein fraction of the feedstock. This ecological role was suggested in previous studies about microalgae digestion (Zamorano-López *et al.,* 2019a, 2019c). *Coprothermobacter* is a common bacteria of thermophilic systems that can be used as a continuous source of thermostable hydrolytic enzymes (Gagliano *et al.,* 2015). The hydrolyzed compounds from microalgae and primary sludge would be further reduced in the $AnMBR_{AD}$ by a syntrophic network with hydrogen-producing bacteria such as *Defluviitoga* (#004). This bacteria also has high potential for complex and diverse carbohydrate degradation such as xylan or cellobiose releasing acetate, hydrogen and carbon dioxide according to Maus *et al.* (2015). The fermentation of peptides could be related to the presence of *Anaerobaculum* (#007) according to Weiss *et al.* (2008) and the metaproteomic study from Hagen *et al.* (2017). Also, the beta-oxidation pathway of long chain fatty acids (LCFA) could be important in thermophilic co-digestion due to the presence of *Syntrophomonas* (#029) in the system (Ziels *et al.*, 2018).

During thermophilic co-digestion the system was partially inhibited since 455 $mgCH_3COOH \cdot L^{-1}$ were detected. Commonly, low C:N ratios observed in microalgae digestion due to the high protein content of

microalgae can trigger ammonia inhibition with a consequent accumulation of fatty acids such as acetate (Sialve *et al.,* 2009). Also, the LCFA have been identified as main inhibitors during microalgae anaerobic degradation (Ma *et al.,* 2015) and other studies have reported the synergetic co-inhibition of both high concentrations of LCFA and ammonia (Tian *et al.,* 2018). Interestingly, high methanogenic diversity was observed in the thermophilic $AnMBR_{AD}$: *Methanothermobacter* (#011), *Methanosarcina* (#017), *Methanosaeta* (#012) and *Methanoculleus* (#025). These methanogens are common in thermophilic digesters according to a multi-omics study of full-scale systems (Maus *et al.,* 2016). This high diversity could be attributed to the partial inhibition status of the system. The higher the methanogenic diversity, the higher the possibility to overcome and establish the equilibrium after consumption of the accumulated acetate. The presence of these four methanogens in the system suggest that methane could be produced in the $AnMBR_{AD}$ through more than one methanogenic pathway (aceticlastic, hydrogenotrophic or methylotrophic).

c. Mesophilic co-digestion

The established community during mesophilic experimental period had a remarkable $OTU_{0.97}$ presence of carbohydrate fermenters belonging to the *Anaerolineaceae* family (#002 and #023) (McIlroy *et al.,* 2017), besides hydrolytic *Coprothermobacter* (#001) and *Fervidobacterium* (#015), which were previously observed during the thermophilic period and remained in the $AnMBR_{AD}$ with 1.5% and 0.3% relative activity values (Table 8.4). *Clostridium* XI (#034) was another mesophilic microorganism observed which is involved in different fermentation reactions (McIlroy *et al.,* 2017). The identification after BLAST comparison of $OTU_{0.97}$ #006 revealed the 8.1% relative abundance and 4.0% activity of *Mesotoga prima* in the mesophilic $AnMBR_{AD}$. According to Nesbø *et al.* (2019) this genus might be involved in the oxidation of acetate in presence of sulfur forms. Further research should elucidate the potential sulfate-reducing metabolism of *Mesotoga* during anaerobic digestion. Finally, the methanogenesis in the mesophilic $AnMBR_{AD}$ could be associated to two main methanogens:

Methanosaeta (#012) and *Methanoculleus* (#025). The dominance of *Methanosaeta* over other methanogens has been described in the previous studies about microalgae digestion and co-digestion using AnMBRs (Zamorano-López *et al.*, 2019a, 2019b) and linked to a steady state in which methane is mainly produced through acetoclastic reactions.

Conclusions

16S rRNA sequencing of the thermophilic $AnMBR_{AD}$ samples better captured the microbial dynamics and discriminated the non-active background microorganisms compared to the 16S rDNA approach. This was not observed for the mesophilic $AnMBR_{AD}$ samples since 63% of the diversity was shared between feedstock and digestate. Hence, the use of rRNA sequencing approach is recommended to characterize thermophilic communities but not mesophilic. The active thermophilic community was dominated by 17.0% *Coprothermobacter*, 9.8% *Anaerobaculum* (hydrolytic and fermentative bacteria) plus 13.9% *Methanothermobacter* and 4.8% *Methanosarcina* which are common thermophilic methanogens. The active mesophilic community had a higher diversity than the thermophilic with 7.7% *Anaerolineaceae* (fermenters), 4.0% *Mesotoga* (potential sulfate-reducer) and dominance of 5.8% *Methanosaeta* (methanogen). The higher methane yield observed under mesophilic conditions (205 $mLCH_4 \cdot d^{-1} \cdot L^{-1}$) was related to a more stable microbial community structure, which was partially inhibited under thermophilic conditions which showed a lower performance (140 $mLCH_4 \cdot d^{-1} \cdot L^{-1}$) and a higher diversity of methanogens.

Contributions

NZL: conception and design, analysis and interpretation of the data, drafting of the article, collection and assembly of data. RSG: start-up, operating and monitoring of the bioreactor and critical revision of the article for important intellectual content DA: statistical expertise. DA and LB: critical revision of the article for important intellectual content, analysis and interpretation of the data. AS: provision of study materials or patients and obtaining of funding. All authors: final approval of the article

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9. Overall discussion

9. Overall discussion

This research work aimed at deepening the understanding of the microbial ecology of anaerobic digestion processes for bioenergy production from renewable sources that are generated in municipalities or that result from the treatment of sewage. The initial hypothesis of this work stated that microbial communities can be shaped until achieving a high specialization for disrupting a certain substrate, including a robust and recalcitrant feedstock such as microalgae. This was demonstrated by different authors (Greses *et al.,* 2017; Klassen *et al.,* 2017) and has been corroborated under different combinations of operational conditions in this work. Since the capacity to degrade microalgae and complex substrates without the application of pre-treatments is associated to the enhancement of a hydrolytic community in anaerobic digesters, the study of the microbial population appears as a valuable step to better understand the performance of these systems. For this purpose, high-throughput sequencing has been applied in the five chapters that compose this dissertation.

9.1.Overview

The core of this dissertation reports different anaerobic systems working in parallel for the same purpose: producing biogas from a source of microalgae harvested from a photobioreactor treating sewage (Chapters 5, 6, 7 and 8). Also, the microbial community of different co-digesters at plant scale has been explored in Chapters 4 and 8. Hence, this work is a good example of how molecular ecology studies can be integrated during the development of technical solutions for resource recovery on a circular economy frame. Chapter 8 especially focuses on the practices that should be improved to better determine the active microbial community of the anaerobic systems. The different combinations evaluated in the study have covered different ranges of temperature from mesophilic (28-38ºC) (Chapters 4, 6, 7 and 8) to thermophilic (50-55ºC) (Chapters 5 and 8). Not only that but also conventional configurations of anaerobic digesters with high SRT capacity have been evaluated and expanded with the coupling of external membrane tanks. The AnMBR configuration has allowed exploring

the effect of high SRT of 50, 70, and 100 days during the acclimation of microbial communities for raw microalgae digestion and also co-digestion (Chapters 6, 7 and 8). Besides, the selection of natural sources of hydrolytic communities such as the rumen from a goat has been evaluated (Chapter 6).

This study started with a learning period about high-throughput sequencing and bioinformatics to integrate the identification and quantification of key microbial groups using these approaches in continuous processes at both laboratory and plant scale levels. After evaluation of different next-generation sequencing technologies, Illumina was chosen as the most convenient sequencing alternative due to its high resolution in terms of retrieved sequences and decreasing cost. The first sequencing analysis was performed over an AnMBR plant treating urban sewage and food waste (Chapter 4). A custom bioinformatics pipeline provided by the genomic service of *Fundación para el Fomento de la Investigación Sanitaria y Biomédica de la Comunitat Valenciana* (FISABIO) was used to analyze the sequences retrieved from the microbial samples collected in the AnMBR plant. This approach revealed the maintenance of a hydrolytic microbial population in the AnMBR after ceasing the addition of food waste. The application of next-generation sequencing revealed the diversity of both *Bacteria* and *Archaea*, that had not been identified in the system before since the studies were limited to the application of fluorescence *in-situ* hybridization (FISH) (Giménez *et al.*, 2011). These findings suggested the potential of microbial communities to adapt to certain substrates and increase the hydrolytic capacity of bioengineered systems, thus motivating the use of high-throughput sequencing techniques in further studies, which are the core of this dissertation (Chapters 5, 6 and 7).

The core of this thesis is composed of the three studies that evaluate the acclimation of different sources of inoculum such as thermophilic digestate (Chapter 5), rumen (Chapter 6) and mesophilic digestate (Chapter 7) to degrade raw microalgae (Chapters 5, 6 and 7) or co-digestion with primary sludge (Chapter 8). Similar communities were observed, but also several differences were identified between the microbial structures promoted under the different operational conditions applied to each system in the different

chapters. Indeed, this is a remarkable aspect of this work, in which different biostatistics analysis have been implemented into the analysis of microbial communities, including NMDS, ANOSIM, PCoA, PLS and PLS-DA. In this way, the significant effect of certain operational parameters such as temperature, SRT, HRT, reactor configuration and OLR has been determined among the different chapters.

9.2. Standardization of methodologies is a required step towards the implementation of microbial ecology in anaerobic digestion models

Nowadays, the region most common in microbial ecology studies applied to anaerobic digestion is the v3 to v4 (Klindworth *et al.*, 2013) since they provide a good coverage and phylum spectrum and reduce the bias during library preparation. In this study, two regions of the 16S rRNA gene have been analyzed through Illumina high-throughput sequencing. The election of the hyper-variable region v3 to v4 (Chapters 5, 6 and 7) was preferred over region v4 (Chapters 4 and 8) due to the detection of a higher diversity of *Bacteria* microorganisms than *Archaea*. The choice of prokaryotic primers has an effect over microbial ecology studies (Tremblay *et al.*, 2015) and therefore they must be carefully chosen, as well as the bioinformatics pipeline used in downstream analysis (López-García *et al.*, 2018)

A custom pipeline including different algorithms in bash programming language was used in Chapter 4 to filter the raw sequences and to extract clean reads. The clean sequences merged according to a certain level of similarity (80%) were classified in Chapter 1 using the Ribosomal Database Project (RDP), which contains taxonomic information according to the 16S rRNA gene of both *Bacteria* and *Archaea*. For Chapter 4 a closed-reference assignment of the sequences to the prokaryotic taxonomy was performed using the RDP gold database. The core of this dissertation (Chapters 5, 6 and 7) was performed using the open-source pipeline QIIME (v1). This allowed the definition of clusters of sequences with more than 97% similarity, which were defined as $OTU_{0.97}$ that allowed the identification of some groups at the species level in the systems using the SILVA database, which is the most updated source of 16S rRNA gene information (López-García *et al.*, 2018).

In these chapters an open-reference approach was used to pick the $OTU_{0.97}$ which allowed to identify clusters of sequences equivalent to $OTU_{0.97}$ that are not present in the reference databases. The experimental phase of Chapter 8 took place at the University of Michigan. For this experiment Mothur was the open-source bioinformatics pipeline used. As in Chapter 4, the taxonomical assignment was performed according to the RDP database. However, the sequences were clustered into $OTU_{0.97}$ as in Chapters 5, 6 and 7 to identify the taxonomy at the species level. Although both Mothur and QIIME bioinformatics pipelines include the algorithms used in Chapter 4, the procedures followed in Chapters 5, 6, 7 and 5 were more practical and also allowed the implementation of additional quality controls.

In Chapter 8 a mock community of the hyper-variable region of the 16S rRNA gene was sequenced within the bioreactor samples. This determines that no bias were produced during library preparation, sequencing or classification. Also, a negative control of the extraction procedures was included in the sequencing project. During library preparation before the sequencing run, no libraries were retrieved from the negative control samples. Hence, this practice determined that all the reads were representative from the original biomass samples and did not resulted from contamination of the reagents or the hands-on procedures. The use of mock communities and negative controls of the extraction in future studies should be considered since it can distinguish natural variations from serious errors (Yeh *et al.*, 2018). The pipeline of Mothur developed by Kozich *et al.* (2013) allows the filter of the sequences including a mock community and thus implies an improved quality level of the downstream sequencing analysis. Moreover, several authors have reported the preferable use of Mothur over QIIME since the first better captures the diversity and richness of microbial communities (López-García *et al.*, 2018) and uses *vsearch* algorithm for $OTU_{0.97}$ picking instead of the *usearch* algorithm implemented in QIIME. This is a remarkable difference that had been reported by Westcott and Schloss (2015), who attributed a lower sensitivity of QIIME to reference the closest sequence to an $OTU_{0.97}$. Hence, it can be concluded that the use of Mothur with the implementation of mock community and a negative control are here recommended for future studies of microbial ecology.

9.3. Microalgae-degrading communities are shaped by temperature, inoculum source and solids retention time

Due to the abovementioned differences in the methodology applied for each chapter, the discussion about the operational parameters influence over microbial communities is mainly performed over the core of this dissertation (Chapters 5, 6 and 7). The samples used in these studies have been merged into a single set to provide a global view of the two most determined operational parameters evaluated in this work: temperature and SRT. The structure microbial communities retrieved from the bench-scale bioreactors are analyzed in a PCoA of the UniFrac distance matrix (Figure 9.1a). A 57% of the variability observed between samples is explained due to the temperature conditions applied to each study, related to the first component of the analysis. An additional 15% of variability is explained in the second component that would be mainly attributed to differences in the inoculum source due to the use of rumen (Chapter 6). However, the effect of operating at high SRT over the microbial community seems to merge the microbial diversity structures observed among the different chapters (Figure 9.1b). Hence and in this order, the temperature, the inoculum source and the SRT would be the three main factors determining the microbial diversity that is enhanced for a common feedstock, since the biomass used to fed the systems of Chapters 5, 6 and 7 was generated in the same photobioreactor and collected in parallel.

As pointed out in Chapter 7, different microbial communities are established during microalgae digestion than during co-digestion. However, a 57% of the diversity is shared between both processes and regardless of the composition of the dominant microalgae. Moreover, the PCoA analysis shows a similarity between the samples taken at short SRT of 7-28 days in Chapter 6 (Figure 9.1b). This could be related to the detection of the microbial diversity associated to the feedstock since the samples collected and a poor status of specialization of the community. In contrast, the remaining samples collected in Chapter 6 at 70-100 days SRT are separately clustered. This highlights the importance of the inoculum source. However, as discussed in Chapter 6, after a 2-fold increase of the OLR the microbial structure of the rumen system shifted. According to Figure 9.1a and 9.1b,

the resulting population is more similar to the rest of the mesophilic systems analyzed in this thesis than to the community that had been promoted from the rumen source. This also confirms that the slightly higher temperature of 39ºC applied in Chapter 6, compared to 35-36ºC of the Chapter 7 systems, was not determinant to shape in a different way the microbial population. Hence, these findings suggest a common trend of systems with different characteristics promoted by the addition of a common feedstock. Despite the distinct inoculum sources, the beta-diversity analysis shows a convergence of the microbial communities developed for raw microalgae anaerobic digestion when operating at mesophilic temperature between 35-39ºC.

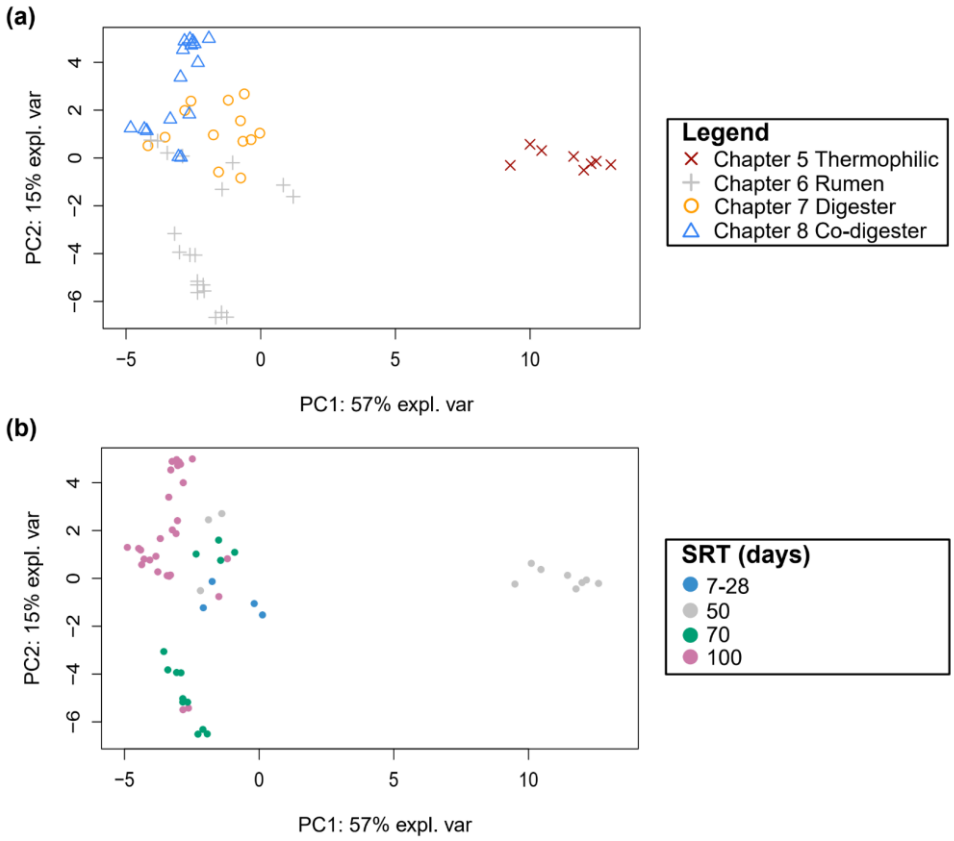

Figure 9.1. Principal co-ordinate analysis (PCoA) of the (a) microalgae degrading systems used in Chapters 2, 3 and 4 and (b) their corresponding solids retention times (SRT).

9.4.Microbial communities for bioenergy recovery

Among the different chapters, a higher diversity has been observed in mesophilic systems than in thermophilic. At the bench scale, 3000-4000 $OTU_{0.97}$ were observed in average in the mesophilic systems (Chapter 7) but only 1400-2500 $OTU_{0.97}$ were detected in the thermophilic systems (Chapter 5). This mainly results from the high selective pressure of the temperature over microbial populations. The sequencing approach in the plant revealed similar results from rRNA in which approximate 500 $OTU_{0.97}$ were detected during pseudo-steady state thermophilic conditions and ~1000 $OTU_{0.97}$ were identified at the mesophilic period. This 2-fold higher diversity of mesophilic systems compared to thermophilic could not be corroborated using rDNA in the plant (Chapter 8) due to the noise effect of non-active microorganisms in the thermophilic plant which resulted in similar observed $OTU_{0.97}$ average values between 900 (thermophilic) and 1000 (mesophilic).

A description about the similarities and differences of microbial composition according to the assigned taxonomy is listed below for Chapter 8 and the abovementioned chapters (Chapters 5, 6 and 7). This analysis is relevant from an up-scale perspective, since Chapter 8 was performed at the plant level while the rest of the chapters correspond to bench-scale studies. Hence, this comparison provides further information about the feasibility of applying direct acclimation of microbial communities in systems for bioenergy recovery. As described in Chapter 8, the AnMBR plant used is integrated in an innovative layout for resource recovery from sewage (Seco *et al.*, 2018).

9.4.1. Themophilic conditions

Similarities in the microbial community were observed between the bench-scale CSTR microalgae digester (Chapter 5) and the $AnMBR_{AD}$ plant co-digester (Chapter 8). The identification the two main phyla was consistent with different distributions between the plant and the bench-scale resulting in 6-44% *Thermotoga*, 17-32% *Firmicutes* average presence. The dominance of *Coprothermobacter* in both systems was observed at similar values. In the thermophilic CSTR this genus ranged 14.9-35.6%. Its presence at the plant level was corroborated through both 16S rRNA and

16S rDNA sequencing: 20.3% and 17.0% mean values. The presence of the group EM3, related to *Thermotoga* phylum and more recently to Candidate phylum *Hydrothermae* was not observed in any thermophilic sample retrieved from the $AnMBR_{AD}$. This could be related to the addition of an additional substrate such as primary sludge or to the sequencing approach since different hyper-variable region was used in Chapter 5 than in Chapter 8.

As pointed out in the comparison between mesophilic microalgae digesters and co-digesters (Chapter 7), the addition of primary sludge had a remarkable effect over microbial community structures. This affected both the species distribution and the composition *i.e.* alpha and beta diversity estimators were different for the digester and co-digester communities. Hence, the co-digestion could also explain the differences observed between Chapter 8 and Chapter 5. For example, the phylum *Proteobacteria* had 12.9% relative abundance and activity 22.1% (mean values) in the $AnMBR_{AD}$ (Chapter 8) but it was part of the minor phyla found in the thermophilic CSTR (Chapter 5). Nevertheless, other factors such as the use of an $AnMBR_{AD}$ and the consequent decoupling of the low HRT (30 days) from the high SRT (70 days) could have contributed to the differences in the microbial population observed between the bench and plant scales. In contrast, these changes would not be related to the change in the microalgae since it has been observed that microbial communities for *Scenedesmus* and *Chlorella* anaerobic digestion have similar microbial structures (Chapter 7) and their species composition has been also reported in other studies using these *Chlorophyta* microalgae (Greses *et al.*, 2017; Klassen *et al.*, 2017; Sanz *et al.*, 2017). Besides, other differences could be related to the exposure to higher environmental thermal variations than the laboratory bioreactors. Despite the temperature control applied in the $AnMBR_{AD}$ plant (Chapter 8), ±1.5ºC system temperature fluctuation was observed. In this context, other microorganisms with faster kinetics and affinities for substrate could have been more promoted in the plant than in the bench-scale reactor.

Regarding the dominant methanogens classified under *Euryarchaeota* phylum, thermophilic non-cultured *Methanosaeta* $OTU_{0.97}$ were detected in

both bench- (Chapter 5) and plant- (Chapter 8) scale systems. However, a higher diversity of methanogenic $OTU_{0.97}$ was observed in the $AnMBR_{AD}$ (*Methanothermobacter*, *Methanosarcina*, *Methanoculleus*, and *Methanosaeta*) than in the thermophilic CSTR (*Methanosaeta* and an uncultured *Methanobacteriaceae* member). It should be highlighted that the microalgae were co-digested in Chapter 8 plant but single digested in the Chapter 5. Hence, the synergies promoted by the combination of two substrates (microalgae and primary sludge) could also contribute to the higher metabolic diversification observed at plant scale. Related to this, *Syntrophomonas* and *Defluviitoga* were detected in Chapter 8 but not in Chapter 5. The first bacteria is known to release hydrogen during beta-oxidation of the LCFA (Hatamoto *et al.,* 2007) while the second has been proposed as a hydrogen scavenger (Fontana *et al.,* 2018). These findings could have contributed to the higher diversity of methanogens observed in the thermophilic $AnMBR_{AD}$ plant (Chapter 8), compared to the bench-scale thermophilic CSTR (Chapter 5).

Finally, the values of relative abundance and activity in Chapter 8 for the thermophilic conditions showed that despite the low detection through 16S rDNA sequencing techniques of *Archaea*, their relative activities are remarkable. For example, *Methanothermobacter* was detected at a low relative abundance of 1.3% in the $AnMBR_{AD}$ using the 16S rDNA as a target. In contrast, 13.9% relative active of this methanogen was detected sequencing the 16S rRNA. Also, higher values of relative activity than abundance were retrieved for the other three methanogens detected in the thermophilic plant. This could be attributed to: (i) a higher presence or importance in metabolic reactions during thermophilic anaerobic digestion, but also to (ii) the reduction of the background community by targeting the 16S rRNA, (iii) higher sensitivity of the primers used in Chapter 8 than those used in Chapter 5. This has been previously discussed in this chapter, but it is worth highlighting again that the election of the primers for high-throughput sequencing studies must be carefully evaluated towards accurate profiling of microbial communities (Ghyselinck *et al.,* 2013; Pinto and Raskin, 2012).

9.4.2. Mesophilic conditions

More mesophilic systems (30-40ºC) have been evaluated in this study since they have an advantage over thermophilic systems in terms of economic and energetic demands savings. This is an important aspect towards the implementation of sustainable technologies focused on the resource recovery from renewable sources. The most relevant phyla were 2-19% *Bacteroidetes,* 15-30% *Chloroflexi*, 2-15 % *Firmicutes,* 14-27% *Proteobacteria* and 1-7% *Synergistes*. The core of mesophilic microalgae digestion is elucidated in Chapter 7 from the dominant $OTU_{0.97}$ detected through 16S rRNA gene sequencing and highlights the importance of bacteria members from *Anaerolineaceae* (main family of the *Chloroflexi* detected), *Syntrophobacterales* (order from *Proteobacteria*), and *Synergistaceae* members (main family of *Synergistes*); besides the methanogens classified in the *Methanosarcinales* order, classified under *Euryarchaeota* phyla which accounted for 1-5%. This microbial core was observed in all bench-scale mesophilic systems regardless of the addition of a co-substrate. Moreover, these phyla were dominant in the AnMBR inoculated with rumen and used for raw *Scenedesmus* conversion into biogas (Chapter 6). Moreover, the study performed in the $AnMBR_{AD}$ (Chapter 8) revealed that these microorganisms are not only remarkable abundant (%) but also active. For example, during mesophilic co-digestion of *Chlorella* in the $AnMBR_{AD}$ plant mean values of 7.8% relative abundance and 9.2% activity of *Anaerolineaceae* were observed. This group contains several fermenters as reported in McIlroy *et al.* (2017).

Different syntrophic microorganisms were detected in the laboratory (Chapters 6 and 7) compared to the plant scale levels (Chapter 8). The main syntrophic microorganisms detected in the bench-scale reactors were *Defluviitoga* and *Smithella*, suggesting that the hydrogen released during fermentation (by microorganisms from *Anaerolineaceae*, among others) was consumed by hydrogen-scavenging bacteria such as *Defluviitoga* (Chapters 6 and 7). Under these conditions, intermediate products such as the propionate can be oxidized into acetate through the *Smithella*-pathway (Leng *et al.,* 2018). Moreover, the co-existence of both *Defluviitoga* and *Smithella* with *Methanosaeta* is reported in this study as the main syntrophic

association in microalgae digesters and co-digesters. In contrast, two of the mesophilic systems presented a different syntrophic network. The hydrogen producer *Gelria* was observed in the rumen AnMBR (Chapter 6) and in the mesophilic AnMBR (Chapter 7), both bench-scale bioreactors. In these systems, no other syntrophic microorganisms were identified at remarkable relative abundances. The presence of *Gelria* in Chapter 6 could be also related to the use of rumen as inoculum since *Gelria* was present at low relative abundance in the start of the experiment but was enhanced up to 13.2% relative abundance values during performance.

We hypothesize that the hydrogen potentially released by *Gelria* could be converted into methane by hydrogenotrophic methanogens. The versatile metabolism of *Methanosaeta* which was the dominant methanogen in these systems (Chapters 6 and 7) should be highlighted. This methanogen is capable of cleaving the acetate or release methane through the reduction of carbon dioxide using the hydrogen as an electron donor. Also, it was the dominant methanogen observed in the mesophilic co-digester plant (Chapter 8). Related to this, Sundberg *et al.* (2013) reported that methanogenic metabolism shifts to hydrogenotrophic pathways when operating at higher temperatures or during co-digestion, as in this study. These pathways would include the syntrophic propionate oxidation (Leng *et al.*, 2018) in which microorganisms such as *Smithella* and *Methanosaeta* with a third microorganism involved in the scavenging of hydrogen are involved. This process was pointed out as the presumably main pathway for methane production in the microalgae co-digesters analyzed in our study at both laboratory (Chapter 7) and plant scales (Chapter 8). Furthermore, the dominance of aceticlastic methanogenesis was related to the high biomethanization achieved in the AnMBR plant treating food waste and urban wastewater (Zamorano-López *et al.*, 2018) (Chapter 4). In this case, the concentration of sulfate in the influent of the plant could have contributed to the dominance of aceticlastic methanogenesis instead of syntrophic associations. Sulfate-reducers uptake both hydrogen and acetate released in the previous fermentation stages of anaerobic digestion. As long as high concentration of acetate is available, aceticlastic methanogenesis occurs. The presence of sulfate-reducers in the AnMBR plant ranged 1.5-

4.6% relative abundance values. We hypothesize that hydrogen was quickly scavenged by these microorganisms and used in their heterotrophic growth over acetate (Plugge *et al.,* 2011). However, the methanogenic metabolism was not outcompeted in the AnMBR due to the high influent COD/S-SO_4 ratio reached through the combined anaerobic digestion of urban wastewater and food waste. In contrast, a constant flux of acetate was released during organic matter fermentation, being available for both sulfate-reducers and methanogens and resulting in the high methane yields observed in this AnMBR plant study (144.5 L $CH_4 \cdot kg^{-1}$ influent COD) (Chapter 4).

9.5. Microbial communities for raw microalgae degradation can be quickly promoted from mesophilic digestate and should be preferred over the use of rumen as inoculum

The use of rumen has more technical issues than the use of mesophilic biomass from full-scale digesters. However, the results here reported support the use of both inoculum sources at the laboratory scale (Chapters 6 and 7). As presented in Chapter 6, the microbial community promoted from rumen can provide a quick and efficient degradation of raw microalgae reaching biodegradability values over 60.0%. The efficiency of the process was highly related to the enhance of *Fervidobacterium* from a 0.1-1.8% in the inoculum up to a 26.8%. However, after increasing 2-fold the organic OLR of the system, the microbial structure was affected and *Fervidobacterium* was overpassed by other microorganisms that were also present in the system during the previous periods, but at lower abundances. Interestingly, the microbial profiles observed in the rumen AnMBR at high SRT of 70 days and OLR of 0.4 $gCOD \cdot L^{-1} \cdot d^{-1}$ were similar to the microbial core described for the mesophilic microalgae digesters and co-digester (Figure 9.1). After increasing the OLR in the rumen AnMBR, *Anaerolineaceae* increased 2-fold and more *Proteobacteria* microorganisms, including the syntrophic *Smithella* were enhanced in the system (Chapter 6). This suggests that there is a cross-sectional microbial population at higher SRT of 70-100 days when operating AnMBRs that has the potential to naturally degrade complex feedstocks such as microalgae. This has been reflected in this chapter through PCoA analysis of all the samples analyzed in the core chapters of this dissertation (Figure 9.1). Hence, the use of rumen as an inoculum should

be avoided and replaced by the acclimation of mesophilic full-scale digester biomass since these communities are more robust in terms of structure low fluctuation against changes in the operational conditions.

9.6. The election of rRNA reveals a more accurate characterization of microbial diversity

The previous studies about microalgae digestion and co-digestion in the laboratory scale suggested that a background community remained in the systems when operating at high SRT (Chapter 7). For this reason, a different methodology was applied in Chapter 8 aiming at discriminating between the active and non-active groups in the AnMBR through targeting the rRNA of the digestate and the rDNA of the feedstock. Most of the active members detected through rRNA/rDNA sequencing in Chapter 8 had been previously observed at the laboratory scale in Chapters 5, 6 and 7. In contrast, other microorganisms that were detected in very low abundances targeting rDNA increased their presence in the active profiles retrieved from rRNA and others with high redundancy through rDNA measures were poorly or non-detected through rRNA. These results suggest that the implementation of rRNA/rDNA sequencing improves the elucidation of the key microorganisms that are involved in anaerobic digestion. This approach is here recommended for thermophilic digesters, since there is a clear discrimination between redundant microorganisms detected using rDNA, that might be related to the feedstock and not to the digestate. In contrast, these groups are not detected using rRNA and thus the rRNA approach better captures the microbial diversity of thermophilic systems.

The rRNA approach is not recommended for systems operated at mesophilic conditions. The higher sensitivity of the rRNA sequencing results in inconsistent detection of active groups that are not detected using rDNA. A certain microbial group must be detected through both rRNA and rDNA to obtain an accurate population characterization in microbial ecology studies. Hence, the rRNA analysis of mesophilic systems is not recommended, especially for systems operated at high SRT in which the microorganisms are retained during long periods. Since the cost of rRNA analysis is higher than rDNA, requires longer hands-on periods and higher

knowledge on molecular biology methods; it is not worth. In these cases, a single rDNA approach targeting the 16S rDNA could provide a full vision of the microbial structure of the system.

Finally, rRNA could be a good choice in dynamics studies about the effect of inhibition processes over microbial populations due to its higher detection sensitivity. The low abundant groups play a resilient role in complex communities. Compared to rDNA sequencing, the rRNA approach can better detect the microbial fluctuations during process disturbances. Overall, the election of the sequencing approach must always be carefully evaluated and defined towards the understanding of specific situations or problems in bioengineering systems.

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10. Conclusions and Future Perspectives

10. Conclusions and Future Perspectives

This thesis has focused on the microbial ecology of anaerobic digesters used to recover energy from renewable sources. The main objectives were: (i) to study the enhancement of the hydrolytic microorganisms in different bioreactors at the laboratory and plant scales, (ii) to understand the changes in their microbial structures under different operational conditions, (iii) to evaluate the use of acclimated communities to certain substrates and avoid the application of pre-treatments, and (iv) to explore the influence of the feedstock composition over microbial populations. The main conclusions of this work are listed below:

i. The influence of temperature over microbial communities was higher for thermophilic systems than for mesophilic due to the niche specialization capacity of the microorganisms. Higher bacterial diversity was observed in the mesophilic systems (3000-4000 $OTU_{0.97}$) compared to the thermophilic (1400-2500 $OTU_{0.97}$) at the bench scale systems. These differences were observed at the plant scale using rRNA sequencing but not with the rDNA approach due to the detection of non-active groups in the thermophilic population. The number of observed $OTU_{0.97}$ in the thermophilic plant was 2-fold the mesophilic value.

ii. Different microorganisms with a potential hydrolytic role in anaerobic digesters were detected at both laboratory and plant scales. According to the taxonomy, they were distributed in mesophilic systems as follows: 2-19% *Bacteroidetes,* 15-30% *Chloroflexi*, 2-15% *Firmicutes*, 14-27% *Proteobacteria* and 1-7% *Synergistes*. For thermophilic systems the average distribution was 6-44% *Thermotoga* and 17-32% *Firmicutes*.

iii. The thermophilic systems had similar diversity in terms of species composition with a dominance of 20-35% *Coprothermobacter* (proteolytic bacteria), which had relative actives of 17%. The most

remarkable group under mesophilic conditions was *Anaerolineaceae* (7.8% relative abundance with 9.2% relative activities), in which several fermentative bacteria are classified. These findings support the use of acclimated communities to degrade renewable sources and avoid biomass pre-treatment.

iv. Two microorganisms were specific to certain bioreactors due to their configuration and operational conditions. The first, 26.8% *Fervidobacterium* (hydrolytic bacteria), was only observed in the mesophilic system that was inoculated with rumen. The second, 44.5% *Hydrothermae* EM3, is a novel group related to *Thermotoga* and it was only observed in the thermophilic bioreactor at the laboratory scale. Both microorganisms are here proposed as hydrolytic bacteria specific to microalgae degradation although they have a high sensitivity to operational conditions. *Proteobacteria*, *Bacteroidetes* and *Firmicutes* were present in both feedstock and digestate communities. This analysis of rDNA revealed that the thermophilic digestate microbial community had a redundancy of these microorganisms. However, the rRNA analysis discriminated against the non-active groups and highlighted the effect of temperature over microbial communities. Although the mesophilic systems were operated at different temperatures between 28-39ºC, similar microbial structures were found regardless of the temperature. Furthermore, a 57% shared microbial diversity was found between these mesophilic systems at the laboratory scale. Thus, the differences between mesophilic microbial structures were attributed to other operational conditions such as HRT or SRT.

v. The use of rumen as inoculum allowed the enhancement of an efficient microbial community for microalgae degradation. Other operational conditions such as the higher OLR and the shorter HRT modified the species distribution resulting in lower biomethanization yields. In contrast, microbial communities were quickly promoted from mesophilic digestate and showed high robustness against

changes in operational conditions such as different feedstock composition, SRT, and OLR. Hence, acclimated communities should be preferably selected over exogenous hydrolytic consortia like rumen.

vi. The effect of SRT was remarkable for each system but an overall trend was not observed when comparing all the mesophilic systems at the laboratory scale. This is related to the operation at high SRT. However, this difference was noticeable at low SRT between 7 and 50 days. It can be concluded that other conditions such as the temperature or the inoculum source have a higher pressure over microbial structures especially when operating high solids retention systems.

vii. The food waste degrading community remained in the AnMBR plant in a later period in which only urban was anaerobically digested. This supports the use of the AnMBR system to retain the microorganisms selected during acclimation to specific renewable sources and achieve high biomethanization yields. This was also corroborated when degrading microalgae under stable operational conditions at both laboratory and plant scales.

viii. The composition of the feedstock in terms of microalgae species dominance showed no significant differences at the laboratory and the plant scales. Moreover, a 57% shared diversity was observed between the mesophilic digesters at the laboratory. At the plant level, high stability of the microbial structure was observed despite the fluctuations in the microalgae species fed to the system during mesophilic operation. The robustness of this microbial structure was corroborated using both rDNA and rRNA sequencing approaches.

The novelty of this thesis relies on being the first work evaluating similar systems treating a renewable source obtained in a WRRF to recover energy from urban wastewater and during different operational periods, including

the use of food waste or microalgae to enhance the bioenergy recovery. The sequencing of rDNA has been applied to systems at both the laboratory and the plant scale, finding similarities between microbial community diversity and structure. The role of microbial ecology in the study of bioengineered systems is here highlighted since it has been used to expand our understanding of the effect of operational conditions over microbial communities and therefore, over the biomethanization. From this thesis, future developments are proposed to continue benefiting from the information provided by the study of the microbial communities and moving forward the application of this knowledge in the engineering field:

- Key groups for microalgae or food waste degradation were identified in this work such as *Coprothermobacter*, *Fervidobacterium* o members of the *Anaerolineaceae* famlily or *Hydrothermae* EM3 group. Hence, their use in bioaugmentation is here suggested as a strategy to speed up the acclimation for complex renewable sources. However, some of these microorganisms (like *Anaerolineaceae* members or the novel group *Hydrothermae* EM3) are still uncultured and thus efforts in their isolation would be needed to achieve this goal.

- The study of rDNA in this work has expanded the available sequences in different databases since the ribosomal 16S rDNA was targeted in this study, this information can be compiled and used to design different oligonucleotide probes. These probes could be specific to a certain microorganism (such as *Coprothermobacter* or *Fervidobacterium*) or be merged into a multiprobe that would include relevant bacteria (like *Smithella* or *Defluviitoga*) archaea groups (*Methanosaeta*) for microalgae anaerobic digestion. These groups could be monitored through techniques with a lower cost than sequencing approaches, such as qPCR, droplet digital PCR or FISH.

Appendixes

Appendix A. Nomenclatures and abbreviations

ABR	Anaerobic Baffled Reactor
AcMA	Acetotrophic Methanogens
AD	Anaerobic Digestion
AMPTS	Automatic Methane Potential Test System
AnMBR	Anaerobic Membrane Bioreactor
$AnMBR_{AD}$	side stream Anaerobic Membrane Bioreactor
ANOSIM	Analysis Of Similarities
ANOVA	Analysis of Variance
ASBR	
ASBR	Anaerobic Sequencing Batch Reactor
BMP	Biomethane Potential
bp	Base pair
CA	Canonical Analysis
CAS	Conventional Activated Sludge Systems
CCA	Canonical Correspondence Analysis
cDNA	Complementary Deoxyribonucleotide
COD	Chemical Oxygen Demand
CODr	Chemical Oxygen Demand Removal
CSTR	Continuous Stirred Tank Reactor
CT	Co-Substrate Tank
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycitosine triphosphate
DESASS	Design and Simulation of Activated Sludge Systems
DGGE	Denaturing Gradient Gel Electrophoresis
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleotide
dNTPs	Deoxynucleotide triphosphates
dTTP	Deoxythymidine triphosphate
EGSB	Expanded Granular Sludge Bed Bioreactor
FISABIO	Fomento de la Investigación Sanitaria y Biomédica de la Comunidad Valenciana
FISH	Fluorescence *in-situ* Hybridization
FW	Food Waste

GC-FID	Gas Chromatograph with Flame Ionization Detector
H_2MA	Hydrogenotrophic Methanogens
HRT	Hydraulic Retention Time
LCFA	Long Chain Fatty Acids
MA	Methanogenic Archaea
MANOVA	Multivariate Analysis of Variance
MDS	Multidimensional Scaling
MeMA	Methylotrophic Methanogens
MPBR	Membrane Photobioreactor
MT	Membrane Tank
NMDS	Non-metric Multidimensional Scaling
nt	Nucleotide
ODS	Organic Dry Solids
OLR	Organic Loading Rate
OTU	Operational Taxonomic Units
$OTU_{0.97}$	3% Dissimilarity Operational Taxonomic Units
PBR	Photobioreactor
PCA	Principal Component Analysis
PCoA	Principal Co-ordinates Analysis
PCR	Polymerase Chain Reaction
PERMANOVA	Permutational Multivariate Analysis
PF	Penetration Factor
PLS	Partial Least Square Analysis
PLS-DA	Partial Least Square with Discrimination Analysis
QIIME	Quantitative Insights of Microbial Ecology
R_{ANOSIM}	ANOSIM Statistic R
RDA	Redundancy Analysis
rDNA	Ribosomal Deoxyribonucleotide
RDP	Ribosomal Database Project
RNA	Ribonucleotide
rRNA	Ribosomal Ribonucleotide
RT	Regulation Tank
SAO	Syntrophic Acetate Oxidation
S-COD	Soluble Chemical Oxygen Demand

SMA	Specific Methanogenic Activity
sPLS	sparse Partial Least Square Analysis
SRA	Sequencing Reading Archive
SRT	Solids/Sludge Retention Time
SSU	(Ribosomal) Small Subunit
T-COD	Total Chemical Oxygen Demand
TRFLP	Terminal Restriction Fragment-Length Polymorphism
TS	Total Solids
TSS	Total Suspended Solids
UASB	Up-flow Anaerobic Sludge Blanket Reactor
VFA	Volatile Fatty Acids
VIP	Variable Importance in the Projection
VS	Volatile Solids
WRRF	Water Resource Recovery Facilities
WW	Wastewater
WWTP	Wastewater Treatment Plants
YCH_4	Methane Yield

Appendix B. List of Figures

Appendix C. List of Tables

Appendix D. Resumen Extendido

Antecedentes

El agua es un recurso indispensable para la vida, por lo que su gestión es esencial para garantizar su disponibilidad y mantener un equilibrio entre el uso y calidad de esta. Junto a la escasez de agua, la demanda energética y el calentamiento global asociado a las emisiones de gases de efecto invernadero, son algunos de los principales retos de este siglo. En la Unión Europea (UE) la Directiva Marco de Aguas (2000/60/EC) fue aprobada en el año 2000 por los países pertenecientes con el fin de regular las masas de aguas, su uso y conservación. Además, la UE está adoptando medidas para desarrollar una economía competitiva en términos de producción de energía con bajas emisiones de dióxido de carbono. Esta regulación pone de manifiesto la necesidad de un cambio de paradigma a nivel económico y social, que lleva intrínseca la persecución de un escenario global más sostenible que el actual. Este cambio se ha hecho patente en el ámbito del tratamiento de las aguas residuales. En la actualidad, se están evaluando diferentes estrategias para maximizar la recuperación de recursos a partir del agua residual.

La combinación de procesos anaerobios y microalgas para el tratamiento de aguas residuales establece un nexo entre la recuperación de agua y energía. Esta estrategia se enmarca en la economía circular del agua y es en la que se centra este trabajo. Además, los procesos de digestión anaerobia permiten recuperar energía de otras fuentes de biomasa que son generadas en las áreas urbanizadas (como la fracción orgánica de los residuos sólidos urbanos o FORSU), o durante el tratamiento convencional de las aguas residuales (fangos). El aprovechamiento energético de esta biomasa es llevado a cabo en digestores anaerobios, en los que distintos microorganismos degradan en ausencia de oxígeno la materia orgánica y la mineralizan hasta su forma más reducida. El producto final es un gas de alto contenido en metano (40-70%) y un poder calorífico elevado (6.0-6.5 $kWh \cdot m^3$). La importancia del conocimiento de la microbiología de estos sistemas se pone de manifiesto en este trabajo, puesto que una compleja red de relaciones metabólicas e interacciones entre los distintos grupos de

microorganismos son los responsables de este proceso de recuperación energética. El conocimiento de la ecología microbiana de los procesos anaerobios puede ampliar nuestra capacidad de entendimiento, permitiendo así la optimización de los procesos de una forma más multidisciplinaria, que se acerque más a la naturaleza de estos sistemas (digestores anaerobios).

El desarrollo de técnicas de secuenciación masiva en la última década ha facilitado el estudio de las comunidades microbianas de sistemas complejos y de alta diversidad, tales como los digestores anaerobios. El análisis de DNA permite el estudio de las poblaciones microbianas sin la necesidad de que estas sean cultivadas a nivel de laboratorio, principal cuello de botella. La caracterización microbiológica de distintos sistemas para la recuperación energética a partir de fuentes renovables, operados bajo distintas condiciones operacionales, es el principal objetivo de esta tesis. De esta forma, es posible mejorar el diseño de sistemas a mayor escala en los que se intente aprovechar el potencial de las comunidades microbianas, para reducir los costes de las tecnologías futuras empleadas en la recuperación de recursos como la energía.

Alcances y objetivos

Los objetivos de esta tesis han sido: (i) estudiar el aumento del potencial hidrolítico de las poblaciones microbianas en distintos biorreactores a escala de laboratorio y planta piloto, (ii) comprender los cambios en las estructuras microbianas a través de la evaluación de las distintas condiciones operacionales aplicadas para maximizar la producción de biogás, (iii) evaluar el uso de comunidades microbianas aclimatadas para degradar biomasa de alto contenido energético sin aplicar pretratamientos y (iv) explorar la influencia de la composición de la biomasa sobre las comunidades microbianas de los digestores anaerobios.

Este trabajo se ha estructurado como un compendio de artículos en los que los anteriores objetivos generales se desarrollan de manera específica, tal y como se detalla a continuación:

Capítulo 4

- Desarrollar una metodología para caracterizar comunidades microbianas mediante técnicas de secuenciación masiva de DNA y su posterior análisis con técnicas de bioinformática
- Caracterizar el efecto de la codigestión de la FORSU y el agua residual urbana sobre la comunidad microbiana
- Identificar los microorganismos relacionados con el aumento en la biometanización observado en la planta piloto anaerobia

Capítulo 5

- Caracterizar la comunidad microbiana establecida en un biorreactor termofílico para la recuperación de energía a partir de microalgas sin pretratar
- Explorar el efecto de la velocidad de carga orgánica sobre la estructura de la comunidad microbiana

Capítulo 6

- Evaluar en digestores anaerobios, desde una perspectiva de la ecología microbiana, el uso de biomasa ruminal como fuente microorganismos hidrolíticos
- Identificar las estructuras microbianas establecidas en un biorreactor inoculado con rumen para el tratamiento de microalgas sin pretratar mediante digestión anaerobia
- Analizar los cambios en la población microbiana y su estructura durante la operación a distintas velocidades de carga orgánica

Capítulo 7

- Identificar los principales miembros del microbioma de la digestión y codigestión mesofílica de microalgas
- Comprender las diferencias en la diversidad microbiana y su estructura y su relación con la codigestión de microalgas y fango primario

Capítulo 8

- Elucidar la comunidad microbiana activa establecida durante la codigestión de microalgas en condiciones termofílicas y mesofílicas

- Explorar la influencia de la comunidad microbiana asociada a la biomasa usada como cosustrato sobre la comunidad microbiana del biorreactor y su estructura

Materiales y Métodos

Se ha empleado técnicas de secuenciación masiva para analizar las estructuras microbianas y la diversidad de distintos digestores anaerobios, con distintas configuraciones tales como reactor continuo de tanque agitado (CSTR) (Capítulo 5) o reactor anaerobio de membranas (AnMBR) (Capítulos 4, 6, 7 y 8) que producen bioenergía a partir de diferentes sustratos como microalgas (Capítulos 5, 6, 7), microalgas junto con fango primario (Capítulos 7 y 8), o la fracción orgánica de los residuos sólidos urbanos junto con agua residual urbana (Capítulo 4). La experimentación ha sido llevada a escala piloto en los Capítulos 4 y 8; y a escala de laboratorio en los Capítulos 5, 6 y 7. Las condiciones mesofílicas (28-39ºC) han sido evaluadas en los Capítulos 4, 6, 7 y 8; mientras que las termofílicas (55ºC) se han estudiado en los Capítulos 5 y 8. Las microalgas han sido obtenidas durante toda la experimentación a partir de un fotobiorreactor de membranas a escala piloto que elimina los nutrientes de un efluente de un reactor AnMBR que trata agua residual urbana. Este sistema de recuperación de recursos del agua residual se encuentra ubicado en la instalación de la depuradora municipal "Carraixet" (València) y pertenece al grupo de investigación Calagua Unidad Mixta UV-UPV. La biomasa generada en la planta de microalgas está dominada por dos algas eucariotas, *Chlorella* y *Scenedesmus* y son por tanto los principales géneros estudiados en esta tesis.

La extracción de DNA se ha realizado siguiendo la misma metodología en los Capítulos 4, 5, 6 y 7; ya que tuvo lugar en la Universitat de València. El Capítulo 8 se desarrolló en la University of Michigan y por ello se siguió una metodología diferente, en la que además de DNA se llevó a cabo la secuenciación de RNA. El biomarcador estudiado en todos los capítulos es el gen 16S rRNA y sus transcritos en forma de RNA (Capítulo 8), empleando cebadores para la región v4 (515F-806R) con secuencia *forward 5'-GTGCCAGCMGCCGCGGTAA-3'* y *reverse* 5'-GGACTACHVGGGTWTCTAAT-3' (Capítulos 4 y 8) y la región v3-v4

(341F-806R) con secuencia *forward 5'-CCTACGGGNGGCWGCAG-3'* y *reverse 5'-GGACTACNVGGGTWTCTAAT-3'* (Capítulos 5, 6 y 7). Además, en el Capítulo 8 se incluyó un control de la diversidad detectada por la técnica de secuenciación mediante la preparación de librerías con una comunidad tipo o "*mock community*", cuya composición y abundancias relativas son conocidas. En el resto de los capítulos se incluyeron controles negativos de las extracciones y de las librerías del 16S rRNA secuenciadas.

El procedimiento bioinformático para el análisis de secuencias de DNA desarrollado en el Capítulo 4 fue mantenido en los Capítulos 5, 6 y 7. Esto permitió llegar a identificar los géneros taxonómicos de la comunidad microbiana estudiada. En estos capítulos, de manera adicional, se implementó el uso de QIIME. Esta herramienta de uso libre para el tratamiento de secuencias permitió la detección de OTUs y su asignación en algunos casos a nivel taxonómico de especie. En el Capítulo 8 se trabajó con la herramienta de libre acceso Mothur, que del mismo modo que QIIME, permitió la detección de especies a partir de DNA y RNA. En los Capítulos 5, 6, 7 y 8 se han aplicado distintas técnicas de análisis multivariante para correlacionar la abundancia (Capítulos 5, 6, 7 y 8) y/o actividad (Capítulo 8) de los distintos microorganismos con las condiciones operacionales. De manera más específica:

- el análisis de coordenadas principales (PCoA) evaluó el efecto del aumento de la velocidad de carga orgánica durante la digestión termofílica de algas (Capítulo 5) y en el reactor inoculado con rumen (Capítulo 6), así como la influencia de la configuración del reactor y el HRT-SRT (Capítulo 6) y las distintas composiciones del alimento durante la digestión y codigestión de microalgas o el HRT-SRT (Capítulo 7) sobre la estructura microbiana
- la regresión de mínimos cuadrados parciales (PLS) correlacionó la velocidad de carga orgánica, HRT, SRT y los parámetros fisicoquímicos del proceso con los microorganismos encargados de la degradación de microalgas (Capítulo 6)
- la variante con análisis discriminante del análisis PLS (PLS-DA) se empleó para detectar grupos no abundantes pero discriminantes ante

escenarios de inhibición parcial durante la digestión termofílica de algas (Capítulo 5), o durante la adición o no de un cosustrato (Capítulo 7)
- el análisis multidimensional no métrico (NMDS) se aplicó para explorar el efecto de la temperatura y del cosustrato con la abundancia y actividad relativa de los microorganismos durante la codigestión mesofílica y termofílica de microalgas y fango primario (Capítulo 8)
- el análisis de similitud (ANOSIM) determinó la significancia hipótesis establecidas tras el análisis PCoA (Capítulo 6) y NMDS (Capítulo 8)

Resultados

Entre los principales resultados obtenidos del trabajo, cabe resaltar la puesta a punto de la metodología necesaria para realizar estudios de biología molecular aplicados. En concreto, se seleccionaron los materiales y kits de extracción de ácidos nucleicos más convenientes para las muestras de digestores anaerobios, así como los oligonucleótidos necesarios para llevar a cabo la secuenciación masiva. Además, se adaptaron diversos métodos bioinformáticos para el tratamiento de secuencias obtenidas, con el fin de aumentar la calidad de las secuencias objeto de estudio para así poder evaluar la ecología microbiana de diversos digestores anaerobios.

Durante la adición de FORSU en la planta piloto AnMBR (Capítulo 4) se observó un fuerte cambio en la diversidad poblacional y, asociado a ello, un incremento en el potencial de biometanización del sistema (de 49.2 a 144.5 L $CH_4 \cdot kg^{-1}$ $COD_{entrada}$). Esta mejora del tratamiento anaerobio del agua residual urbana se vinculó a la adición del cosustrato y el efecto sinérgico con la operación a altos SRT que favorecieron la presencia de microorganismos hidrolíticos, fermentadores y metanogénicos en el reactor. Estos microorganismos se clasificaron principalmente en los *phyla Chloroflexi, Firmicutes, Synergistetes* y *Proteobacteria.* La abundancia relativa de los microorganismos hidrolíticos clasificados en *Chloroflexi* y *Firmicutes* aumentaba a medida que una mayor fracción de FORSU era tratada en el sistema AnMBR. Además, el aumento detectado en la actividad metanogénica de 10 a 51 mL $CH_4 \cdot g^{-1}$ VS coincidió con el aumento de la proporción de arqueas del orden *Methanosarcinales* de un 34% a un 80% de abundancia relativa, dentro de este dominio. *Methanosaeta* fue la arquea

metanogénica predominante a la que se atribuye la capacidad de generar metano por la via acetoclástica. Estos resultados permitieron resaltar la capacidad del reactor AnMBR para el desarrollo de comunidades microbianas especializadas en la degradación de una biomasa de alto contenido energético, como es la FORSU. Además, estas estructuras se mantuvieron en el tiempo a pesar de cambios en las condiciones operacionales, tales como el cese de la adición de FORSU, durante más de un periodo equivalente al SRT aplicado, 70 días. Por tanto, este estudio puso de manifiesto la capacidad del AnMBR de mantener en el tiempo las comunidades adaptadas y su uso en distintos procesos de recuperación energética a partir del agua residual.

En los sistemas de digestión de algas (Capítulos 4, 5, 6 y 7) se observaron distintas comunidades microbianas influenciadas por la temperatura de operación y especialmente por el TRC utilizado en el sistema. En los distintos estudios se han alcanzado altos niveles de aclimatación de la biomasa, desde un punto de vista microbiológico, a las diferentes condiciones de operación de cada digestor anaerobio. Por un lado, se ha determinado un grupo central de microorganismos comunes a los distintos digestores de algas independientemente de algunos factores operacionales tales como la carga orgánica o el tiempo de retención hidráulico. Este grupo puede funcionar como un núcleo microbiano que permita hacer un seguimiento de la estabilidad microbiana de un reactor anaerobio produciendo biogás a partir de microalgas o microalgas con fango primario. En este trabajo se revela la composición del grupo, así como su respuesta ante ciertos cambios operacionales. A continuación, se amplía por separado la información recopilada en los distintos capítulos, puesto que fueron desarrollados en sistemas anaerobios diferentes.

La digestión termofílica a escala de laboratorio (Capítulo 5) alcanzó biodegradabilidades de 32-41% a partir de una biomasa algal compuesta principalmente por *Scenedesmus*, que fue digerida sin la aplicación de pretratamientos. El análisis de la comunidad microbiana llevado a cabo a las velocidades de carga orgánica 0.2, 0.3 y 0.4 $g \cdot L^{-1} \cdot d^{-1}$ determinó la dominancia de microorganismos con altas capacidades hidrolíticas tales

como *Thermotogae* y *Firmicutes*, con abundancias relativas de 44.5% y 17.6%, respectivamente además de grupos menos abundantes como *Dictyoglomi*, *Aminicenantes*, *Atribacteria* y *Planctomycetes* (por debajo de 5.5%). Según la información taxonómica y ecológica recopilada en este estudio, estos microorganismos tendrían una gran relevancia en los procesos de hidrólisis de proteínas y fermentación durante la degradación de microalgas. Además, se detectó un grupo nuevo todavía no caracterizado que recibe el nombre de EM3 y que está relacionado con *Thermotogae*. Este grupo estaría hipotéticamente implicado en los procesos de fermentación de compuestos hidrolizados y beta-oxidación de ácidos grasos de cadena larga y, por tanto, tendría una alta relevancia en la producción de metano puesto que los productos finales de su metabolismo sirven como substrato a las arqueas metanogénicas. Además, la aplicación de la técnica PLS-DA resaltó el papel de los géneros *Thermogutta*, *Armatimonadetes* and *Ruminococcaceae* ante un escenario de inhibición parcial de la metanogénesis por acumulación de amoniaco.

La digestión mesofílica a escala de laboratorio empleando un reactor AnMBR inoculado con rumen (Capítulo 6) reveló que la comunidad establecida en estas condiciones estaba dominada por las familias de bacterias hidrolíticas y fermentadoras *Anaerolineaceae*, *Spirochaetaceae*, *Lentimicrobiaceae* y *Cloacimonetes*. Sobre todo, destacó que la elevada abundancia relativa de la bacteria *Fervidobacterium* y *Methanosaeta* se atribuía al 62% de biodegradabilidad observada durante el análisis del proceso de conversión de la biomasa algal de *Scenedesmus* en biogás. El efecto del SRT sobre la comunidad microbiana fue significativo entre las diferentes condiciones aplicadas, pero por encima de ello, se detectó un brusco cambio en la estructura microbiana al duplicar la velocidad de carga orgánica. Ante este cambio, se detectó un aumento de la abundancia relativa de microorganismos sintróficos tales como *Gelria* y *Smithella*.

La digestion y codigestión mesofílica de microalgas y fango primario a escala de laboratorio (Capítulo 7) corroboró el uso de comunidades microbianas aclimatadas para la degradación de microalgas y la producción de biogás. Sin embargo, puso de manifiesto que cuando la biomasa utilizada

como sustrato o cosustrato tiene una alta diversidad microbiana intrínseca, la detección de los microorganismos más relevantes en el proceso de biometanización se vuelve más compleja. Entre los cuatro distintos escenarios posibles para la digestión de algas evaluados, se observó una diversidad microbiana común en el fango del digestor del 57%. Los microorganismos detectados en este núcleo microbiano pertenecían a la familia *Anaerolineaceae*, principalmente, en la que se clasifican distintos fermentadores de polisacáridos. Además, los géneros *Smithella* y *Methanosaeta* fueron observados en los cuatro escenarios, lo que resalta la importancia de la metanogénesis acetoclástica (llevada a cabo por *Methanosaeta*) en sintrofía con la degradación de propionato de *Smithella* y motivó la búsqueda de un tercer microorganismo envuelto en este proceso como consumidor de hidrógeno. La existencia de un tercer implicado en el consumo de hidrógeno garantiza que el propionato continúe reduciéndose a acetato y ser con ello aprovechado por *Methanosaeta*. Este rol fue atribuido a la bacteria *Defluviitoga*, que ha sido propuesta recientemente como consumidora de hidrógeno y cuya abundancia relativa fue correlacionada positivamente con la presencia de *Methanosaeta* y *Smithella*. No obstante, dada la alta diversidad encontrada en estos sistemas, otros microorganismos podrían también haber contribuido al consumo de hidrógeno en este caso.

La codigestión de microalgas y fango primario estudiada a escala piloto (Capítulo 8) reveló que los sistemas termofílicos tenían una diversidad inferior a los mesofílicos. El análisis de 16S rRNA determinó que la comunidad activa tenía una estabilidad menor debido a los cambios observados su estructura mediante el análisis NMDS. Además, el estudio de 16S rDNA sobre el alimento usado como cosustrato (microalgas y fango primario) junto con el análisis de la comunidad del reactor AnMBR determinó que un 63% de la diversidad del alimento se veía reflejada también en la diversidad de la comunidad AnMBR en condiciones mesofílicas. Sin embargo, este hecho no se observó en condiciones termofílicas (14% de diversidad compartida), debido al ya comentado factor de presión selectiva por el cual la mayor parte de microorganismos mesofílicos no prosperan y tampoco permanecen en sistemas a mayor temperatura. La comunidad termofílica activa estaba dominada por las

bacterias *Coprothermobacter*, *Anaerobaculum*, *Fervidobacterium* y las arqueas metalogénicas, *Methanothermobacter* y *Methanosarcina*. En condiciones mesofílicas, se observó la mayor abundancia y actividad de las bacterias *Anaerolineaceae*, *Mesotoga*, *Thermoanaerovibrio* y las arqueas metanogénicas *Methanosaeta* y *Methanoculleus*. Estos resultados corroboraron los resultados obtenidos en previos estudios del biomarcador 16S rRNA, no sólo determinando la presencia de microorganismos similares tanto a escala de laboratorio como de planta piloto, sino que también corroborando su actividad en el sistema mediante la secuenciación de rRNA y rDNA. Por último, este estudio concluyó que el perfil microbiano a correlacionar en futuros análisis conjuntos de proceso y ecología microbiana del sistema AnMBR debería elegir la comunidad descrita a partir de rRNA en condiciones termofílicas y de rDNA en condiciones mesofílicas; dada la reducción en el primer caso de los microorganismos no activos que redundan en el sistema por formar parte del alimento (biomasa algal y fango primario).

Discusión

1. *La estandarización de la metodología es un paso necesario hacia la implementación de la ecología microbiana en el estudio de la digestión anaerobia*

En este trabajo se ha elegido el estudio de la región v3-v4 del biomarcador 16S rRNA en lugar de la región v4 en los Capítulos 4, 5 y 6; debido a su mayor capacidad de detección de diversidad bacteriana sobre la composición de las arqueas. Las secuencias obtenidas fueron representativas de la comunidad microbiana puesto que los controles negativos no permitieron la generación de librerías del biomarcador 16S rRNA.

El uso de QIIME en estos capítulos y de Mothur en el Capítulo 5 permitió la implementación de mayores controles de calidad durante el análisis de secuencias, definiendo las comunidades secuenciadas en base a su composición en OTUs. El uso de una comunidad tipo o "*mock community*" en el Capítulo 8 corroboró la calidad de las secuencias obtenidas tras el filtrado y tratamiento de estas con la herramienta Mothur. De las distintas metodologías evaluadas en esta tesis, se propone el uso de la herramienta de tratamiento de secuencias Mothur para analizar secuencias del 16S rRNA de

bacterias a partir de la región v3-v4, incluyendo controles negativos y comunidades tipo. Sin embargo, la región v4 del 16S rRNA debería ser elegida en caso de querer detectar una mayor diversidad de arqueas y bacterias de manera simultánea. El uso de QIIME es menos recomendable en caso de aplicar una estrategia de selección de OTUs mediante el sistema *open-reference* cuando el objetivo sea la detección de grupos taxonómicos de relevancia. Pese a que esta estrategia tiene un gran poder de detección de nuevos OTUs, a menudo estos no están representados en las bases de datos del biomarcador 16S rRNA y, por tanto, su interpretación ecológica no puede llevarse a cabo. En este sentido, se recomienda la elección de sistemas de selección de OTUs sobre bases de datos filtradas cuyos microorganismos están descritos a nivel taxonómico (sistema *closed-reference*). Esta estrategia puede llevarse a cabo tanto en QIIME como en Mothur y fue la elegida en los Capítulos 4 y 8.

2. *La estructura de las comunidades degradadoras de microalgas derivan de la influencia de la temperatura, el inóculo y el tiempo de retención celular*

Los resultados obtenidos en los Capítulos 4, 5 y 6, que se consideran el núcleo de este trabajo; han permitido elaborar un estudio en conjunto en base a la diversidad encontrada y asociada las distintas comunidades microbianas (Figuras 1a, 1b). Esto es debido a que la metodología utilizada en estos capítulos fue común, a diferencia de la utilizada en los Capítulos 4 y 8. Debido a las razones expuestas en el anterior apartado, estos capítulos han sido excluidos de este análisis conjunto. Este estudio se ha basado en un PCoA de la matriz de distancias UniFrac obtenida a través de las muestras de los cuatro sistemas. Este estudio ha permitido determinar que la temperatura, inóculo y el SRT (en este orden) determinaron la composición microbiana en los digestores de microalgas.

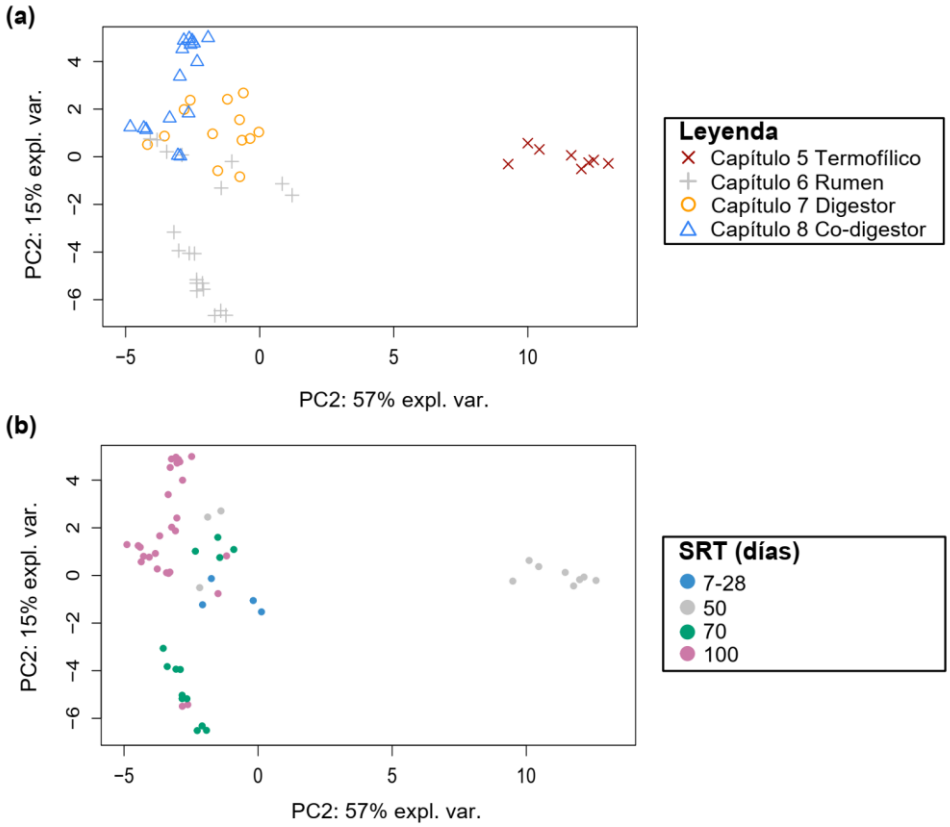

Figura 1. Análisis de coordenadas principales (PCoA) de los (a) sistemas anaerobios de degradación de microalgas estudiados en los Capítulos 4, 5 y 6 y (b) sus correspondientes tiempos de retención celular (SRT).

Tal y como se ha discutido en el Capítulo 7, se establecieron comunidades microbianas distintas en los codigestores que en los digestores de microalgas (Figura 1a) que sin embargo comparten un 57% de su diversidad independientemente del tipo de microalga que se alimenta al sistema. Además, la Figura 1b refleja la similitud entre la diversidad observada en el Capítulo 6 a bajos SRT con la del resto de sistemas. Esto podría estar relacionado con la diversidad asociada a la composición microbiana del alimento y a un bajo estado de especialización de la población. Por lo contrario, las muestras del Capítulo 6 correspondientes a un SRT de 70-100 días se agrupan y separan del resto de muestras mesofílicas del Capítulo 7 (Figura 1a, 1b). Esto señala la importancia del

inóculo puesto que el rumen se utilizó en el Capítulo 6 como fuente de microorganismos hidrolíticos, mientras que en el resto de los capítulos se usó fango de digestores anaerobios municipales. Sin embargo, como se comenta en el Capítulo 6, el aumento de la velocidad de carga orgánica y mantenimiento del SRT a 100 días, supuso un cambio en la estructura microbiana adaptada a las microalgas a partir del rumen. Tal y como refleja el PCoA (Figura 1a), la comunidad resultante se asemeja más al resto de sistemas mesofílicos. Este hecho también confirma que, pese a operar en el Capítulo 6 a una temperatura de 39ºC, no se establecieron diferencias en base a esto sino al inóculo utilizado (rumen), puesto que esta temperatura era ligeramente superior a la del Capítulo 7 (35ºC). Por tanto, se puede concluir que hay una tendencia general relacionada con la composición del alimento que dirige la composición y estructura microbiana hacia una convergencia dentro de sistemas mesofílicos operados entre 35-39ºC. Esta observación se atribuye en este trabajo a la operación a altos SRT de 100 días, que favorece la similitud entre los distintos sistemas.

3. Caracterización de las comunidades microbianas para la recuperación de energía

A continuación, se expone una comparación de las similitudes y diferencias encontradas a nivel taxonómico entre los cinco capítulos que componen esta tesis, con el fin de comparar los trabajos elaborados a escala de laboratorio (Capítulos 5, 6 y 7) y planta piloto (Capítulos 4 y 8) ya que en todos ellos se ha estudiado la ecología microbiana del proceso de digestión anaerobia de la materia orgánica.

a. Condiciones termofílicas

La bacteria *Coprothermobacter* fue observada tanto a escala de laboratorio (Capítulo 5) como de planta piloto (Capítulo 8). Su abundancia relativa en el reactor termofílico CSTR alcanzó el 14.9-35.6%. Esta dominancia fue similar a los valores obtenidos a partir de la secuenciación del rDNA y rRNA en la planta piloto AnMBR (Capítulo 8), con valores de 20.3% y 17.0%, respectivamente. Por lo contrario, la bacteria EM3 que también había sido resaltada en el Capítulo 5, no fue detectada en el Capítulo 8. Esto puede deberse a la secuenciación de una región más corta del

biomarcador 16S rRNA en el Capítulo 8 que en el Capítulo 5, con el consecuente cambio de cebadores. Por otro lado, también podría estar relacionado con la codigestión ya que sólo se evaluó en el Capítulo 8. Precisamente, la adición de fango primario tiene un efecto sobre la diversidad y estructura de las comunidades microbianas, como queda de manifiesto en el Capítulo 7. En base a esto, en la experimentación termofílica de la planta piloto AnMBR (Capítulo 8) se encontró 12.9% de abundancia y 22.1% de actividad relativas de *Proteobacteria*, mientras que este *phylum* fue detectado de manera minoritaria a escala de laboratorio en régimen termofílico (Capítulo 5). No obstante, otras condiciones operacionales derivadas del uso de un sistema AnMBR en el Capítulo 5 que implican separar el bajo HRT (30 días) del alto SRT (70 días) podrían también explicar las diferencias de aparición de *Proteobacteria*. Por lo contrario, estas diferencias no se atribuyen en este trabajo a los cambios en el tipo de microalgas, tal y como se discute en el Capítulo 7. Además, la composición de las comunidades microbianas se asemeja a la reportada por estudios de otros autores en la bibliografía sobre digestión anaerobia de microalgas.

Las arqueas metanogénicas observadas tanto a nivel de laboratorio (Capítulo 5) como de planta piloto (Capítulo 8) correspondieron a OTUs no cultivados del género *Methanosaeta*. Sin embargo, una mayor diversidad de metanogénicas fue observada en la planta piloto (*Methanothermobacter*, *Methanosarcina*, *Methanoculleus*, y *Methanosaeta*). Esto podría estar ocasionado por la codigestión, ya que promueve las sinergias entre los componentes de las microalgas y el fango primerio y el metabolismo de los distintos microorganismos. En relación con esto, *Syntrophomonas* y *Defluviitoga* fueron detectadas en el Capítulo 8 pero no en el Capítulo 5. La primera está involucrada en procesos de degradación de ácidos grasos de cadena larga y la segunda en consumo de hidrógeno. Ambas podrían haber contribuido a la mayor diversidad de microorganismos metanogénicos de la planta piloto (Capítulo 8) puesto que de su metabolismo resultan precursores del metabolismo de producción de metano tales como ácido acético, hidrógeno y dióxido de carbono.

Por último, el análisis de rRNA del Capítulo 8 reveló que pese a detectar arqueas en bajos valores de abundancias relativas como por ejemplo 1.3% de *Methanothermobacter* (Capítulo 8), sus actividades relativas son muy elevadas (13.9% para esta arquea). Esto podría deberse a: (i) la mayor presencia o importancia de sus reacciones metabólicas y, con ello, su actividad; (ii) la reducción en la detección de microorganismos redundantes al secuenciar rRNA, y (iii) mayor sensibilidad de los cebadores usados en el Capítulo 8. Estos resultados ponen de manifiesto que la elección de los cebadores es crucial a la hora de definir los perfiles de las comunidades microbianas de interés.

b. Condiciones mesofílicas

El núcleo microbiano definido en el Capítulo 7 para la digestión mesofílica de microalgas tiene similitudes con los grupos dominantes encontrados en el reactor inoculado con rumen para la digestión de algas (Capítulo 6) y en el codigestor de microalgas y fango primario a escala piloto (Capítulo 8). Miembros de *Anaerolineaceae*, *Syntrophobacterales*, y *Synergistaceae* (*Bacteria*), y metanógenos clasificados en el orden *Methanosarcinales* (*Archaea*) fueron encontrados en estos sistemas. Además, el análisis de rRNA (Capítulo 8) corroboró la relación entre su abundancia y actividad.

Respecto a los microorganismos sintróficos, en los sistemas mesofílicos se observó la presencia de distintos miembros. Por ejemplo, la batería productora de hidrógeno *Gelria* sólo se identificó a nivel de laboratorio en el Capítulo 6 y en el digestor mesofílico AnMBR (Capítulo 7) pero no en los codigestores mesofílicos, a escala de laboratorio o de planta piloto. Por tanto, este microorganismo se relaciona con el uso de un inóculo distinto puesto que se encontró en valores bajos de abundancia al inicio del experimento del Capítulo 6 y llegó a aumentar su abundancia relativa a un 13.2%.

4. *Las comunidades microbianas para la degradación de microalgas sin pretratamientos pueden ser potenciadas con rapidez a partir de fangos mesofílicos, siendo una mejor alternativa que el uso de rumen como inóculo*

El uso de rumen implica un mayor número de consideraciones técnicas que el uso de biomasa anaerobia de digestores a escala industrial. Sin embargo, los resultados de este trabajo apoyan el uso de ambos inóculos a escala de laboratorio (Capítulos 6 y 7). Tal y como se pone de manifiesto en el capítulo 6, la comunidad microbiana que se potencia a partir del rumen proporciona una rápida degradación de algas sin pretratar con altos rendimientos de biodegradabilidad superiores al 60%. La eficiencia del proceso se relacionó con el aumento de *Fervidobacterium* desde 0.1-1.8% en el inóculo hasta 26.8%. No obstante, tras duplicar la velocidad de carga orgánica del sistema, la estructura microbiana se vio afectada y otros grupos de microorganismos desplazaron la población de *Fervidobacterium*. Estos grupos estaban presentes en previas fases del sistema, aunque a menores abundancias relativas. En concreto, los perfiles observados en el AnMBR trabajando a 70 días de SRT y una OLR de 0.4 $gCOD \cdot L^{-1} \cdot d^{-1}$ fueron similares al núcleo microbiano detallado en los digestores y codigestores de microalgas mesofílicos (Figura 1). Tras aumentar la OLR del sistema AnMBR inoculado con rumen, *Anaerolineaceae* duplicó su presencia en el sistema y se detectaron más miembros del grupo *Proteobacteria*, incluyendo al microorganismo sintrófico *Smithella* (Capítulo 6). Esto sugiere que hay una población microbiana que transversal a altos SRT entre 70-100 días cuando se operan sistemas AnMBR, que tienen un potencial natural de degradación de alimentos complejos como las microalgas. Esto se ha reflejado en este capítulo mediante el análisis PCoA de todas las muestras analizadas en el núcleo de este trabajo (Figura 1). Por consiguiente, el uso del rumen como inóculo debería ser evitado y sustituido por la aclimatación de fangos anaerobios de digestores a escala industrial puesto que estas comunidades son más robustas y presentan una menor fluctuación como respuesta a cambios en las condiciones operacionales.

5. *La secuenciación de RNA revela con mayor precisión la diversidad microbiana.*

Los estudios previos sobre digestión y codigestión de microalgas a escala de laboratorio sugirieron que una comunidad "de fondo" permanece en los sistemas cuando se opera a altos SRT (Capítulo 7). Por esta razón, una metodología distinta fue aplicada en el Capítulo 8 con el fin de discriminar entre los grupos activos e inactivos del sistema AnMBR, mediante la detección del rRNA del fango y el rDNA del alimento. La mayoría de los microorganismos activos detectados mediante la secuenciación de rRNA/rDNA en el Capítulo 8 había sido detectada previamente a escala de laboratorio en los Capítulos 5, 6 y 7. Por lo contrario, otros microorganismos que fueron detectados a abundancias muy bajas mediante rDNA aumentaron su presencia en los perfiles de microorganismos activos obtenidos mediante la secuenciación de rRNA. Además, otros con alta redundancia en base a la detección mediante rDNA fueron detectados en valores muy bajos o incluso no detectados mediante secuenciación de rRNA. Estos resultados sugieren que la implementación de la secuenciación de rRNA/rDNA mejora la caracterización de los microorganismos claves involucrados en la digestión anaerobia. Esta estrategia es recomendada especialmente para digestores termofílicos, puesto que hay una clara discriminación entre los microorganismos redundantes detectados mediante rDNA, que pueden estar relacionados con el alimento y no con el fango. Por contraposición, estos grupos no se detectan mediante secuenciación de rRNA y por tanto, esta es una mejor metodología para capturar la diversidad microbiana de sistemas termofílicos.

La secuenciación de rRNA no se recomienda para sistemas operados en condiciones mesofílicas. La mayor sensibilidad de esta técnica proporciona una detección de grupos aparentemente activos, pero que no son identificados mediante la secuenciación de rDNA. Con el fin de obtener un perfil representativo de una comunidad microbiana, un grupo microbiano debe ser detectado mediante las dos secuenciaciones: rRNA y rDNA. Por tanto, el análisis de rRNA en sistemas mesofílicos no se recomienda, especialmente en sistemas operados a altos SRT en los que los

microorganismos son retenidos durante largos periodos de tiempo. Esta práctica no es aconsejable en estos casos, puesto que el coste de la secuenciación de rRNA es más elevado que el de rDNA, requiere una mayor dedicación manual y técnica en el laboratorio y un mayor conocimiento de la biología molecular. Sin embargo, la secuenciación del gen del 16S rRNA mediante rDNA proporciona una visión completa de la estructura microbiana del sistema.

Por último, el análisis de rRNA podría ser una buena estrategia a la hora de estudiar las dinámicas poblacionales, por ejemplo, durante procesos de inhibición sobre comunidades microbianas dada su mayor sensibilidad. Los grupos de bajas abundancias juegan un papel de resiliencia en las comunidades microbianas complejas. En comparación con la secuenciación de rDNA, el estudio del rRNA puede detectar mejor las fluctuaciones microbianas durante problemas operacionales. Por encima de todo, la elección de la estrategia de secuenciación debe ser siempre elegida cuidadosamente, evaluada y definida hacia la comprensión de situaciones específicas o problemas de los sistemas de bioingeniería.

Las principales conclusiones de este trabajo son:

i. El rango de temperatura es el parámetro con la mayor influencia sobre las comunidades microbianas. Los *phyla* más destacados de los sistemas mesofílicos y sus abundancias relativas fueron 15-30% *Chloroflexi*, 14-27% *Proteobacteria*, 2-19% *Bacteroidetes*, 2-15% *Firmicutes*, y 1-7% *Synergistes*. En los sistemas termofílicos destacaron 17-32% *Firmicutes* y 6-44% *Thermotoga*.

ii. Los sistemas mesofílicos de degradación de microalgas compartían un 57% de su diversidad microbiana. Las diferencias observadas fueron atribuidas a los tiempos de retención hidráulico y celular. El alcance de altos tiempos de retención celular facilita la especialización de los microorganismos al alimento y para ello, se recomienda el uso del reactor AnMBR. Las microalgas *Scenedesmus* y *Chlorella*, que crecen

en efluentes anaerobios, pueden ser degradadas por comunidades microbianas similares y sin la aplicación de pretratamientos.

iii. El uso de comunidades aclimatadas debe ser elegido preferentemente antes que el uso de cultivos externos hidrolíticos, ya que tienen una mayor resistencia ante cambios en las condiciones operacionales durante la optimización de los procesos de producción de biogás.

iv. La alta biodegradabilidad de microalgas sin pretratamientos fue atribuída a la abundancia de dos microorganismos específicos en dos sistemas diferentes a escala de laboratorio, *Fervidobacterium* (27%) y el grupo nuevo *Hydrothermae* EM3 (44%). Sin embargo, estos grupos demostraron una alta sensibilidad a los cambios en las condiciones operacionales que afectaron al rendimiento en términos de producción de biogás.

v. El análisis de rDNA/rRNA se recomienda para sistemas termofílicos con el fin de eliminar los grupos de microorganismos de fondo que se asocian a la diversidad microbiana intrínseca de la biomasa. Esta técnica reveló que a escala planta piloto había una destacada presencia/actividad de 20%/17% *Coprothermobacter* y 8%/9% *Anaerolineaceae*, durante la codigestión de microalgas en condiciones termofílicas y mesofílicas, respectivamente.