Ph.D. Thesis Rebecca Serna García

ANAEROBIC CO-DIGESTION OF MICROALGAE AND PRIMARY SLUDGE IN AN ANAEROBIC MEMBRANE BIOREACTOR FOR RESOURCE RECOVERY (BIOGAS AND BIONUTRIENTS) FROM URBAN WASTEWATER

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D. **ALBERTO BOUZAS BLANCO**, profesor titular del Departamento de Ingeniería Química de la Universidad de Valencia y,

Dña. **AURORA SECO TORRECILLAS**, catedrática del Departamento de Ingeniería Química de la Universidad de Valencia,

CERTIFICAN:

Que la presente memoria, que tiene por título: "*Anaerobic co-digestion of microalgae and primary sludge in an anaerobic membrane bioreactor for resource recovery (biogas and bionutrients) from urban wastewater*" corresponde al trabajo realizado bajo su dirección por Dña. **REBECCA SERNA GARCÍA**, para su presentación como tesis doctoral en el programa de doctorado de Ingeniería Química, Ambiental y de Procesos de la Universitat de València.

Y para que conste, firman el presente certificado en Valencia, a 15 de octubre de 2020.

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Abstract

Continuous primary energy consumption has motivated the scientific community to search for less resource-demanding technologies and alternative renewable and eco-friendly energy sources that could substitute fossil fuels. Microalgae cultivation in combination with anaerobic technologies arose as a promising and sustainable technology in the field of wastewater treatment. Microalgae biomass valorisation through anaerobic digestion (AD) allows energy production from waste streams. Since microalgae AD presents some drawbacks that hinder process efficiency, anaerobic co-digestion (ACoD) with carbon-rich substrates such as waste paper or sludge has been studied as an alternative to increase anaerobic process efficiency.

The present study consists in the long-term evaluation of raw microalgae and primary sludge ACoD. For this purpose, the ACoD technology was included in an environmentally sustainable framework in which AD was used first for organics removal from settled raw wastewater; microalgae cultivated in a membrane photobioreactor pilot plant were used for nutrients removal from the AD effluent; and microalgae biomass and primary sludge were co-digested in an anaerobic membrane bioreactor (AnMBR). Microalgae and primary sludge were co-digested at laboratory and pilot-scale and different operating conditions were evaluated considering biological processes, membrane filtration and microbial community involved.

Microalgae and primary sludge ACoD was studied first at lab-scale, operating at different solids retention time (SRT), organic loading rate (OLR) and temperature. Results indicated that, the higher the SRT and the OLR, the higher the methane production, working in an AnMBR. Laboratory results also showed that thermophilic AnMBR achieved higher methane yield than the mesophilic one. Nevertheless, economic and energetic balances carried out to know which are the best operating conditions to scale up the process from laboratory to pilot-scale showed that 70 d SRT and mesophilic conditions were the recommendable ones to operate the ACoD pilot plant. Then, based on these results, the ACoD pilot plant was operated during a year at 70 d SRT and 35 ºC. The pilot plant AnMBR achieved 62% organic matter biodegradability and showed high stability in terms of pH and volatile fatty acids. Filtration process was assessed, indicating

that applying gas sparging and a backwash cycle every two filtration cycles avoided irreversible fouling formation.

Microalgae ACoD has demonstrated to increase methane production compared to microalgae mono-digestion. Adding an easily biodegradable substrate as primary sludge to microalgae biomass had a synergetic effect on AD, creating an adapted microbial community capable of degrading both substrates through syntrophic pathways in which syntrophic microorganisms as *Smithella* or *W5* degrade intermediates (especially propionate) and enhancing methane production, mainly carried out by aceticlastic methanogens as *Methanosaeta*. Hydrolytic and fermenters microorganisms involved in protein degradation (e.g. *Coprothermobacter*, *Fervidobacterium*, members of Synergistaceae family) and cellulose degradation (e.g. *Defluviitoga*, *Thermogutta*) also had an important role in both substrates degradation.

This work also assessed potential nutrient recovery from ACoD effluents (permeate and digestate). Nitrogen was recovered from permeate with 99% efficiency using a hydrophobic polypropylene hollow-fibre membrane contactor. Phosphorus was not recovered from permeate since 74% was precipitated during AD process. Laboratory experiments with ACoD digestate carried out in Dewar reactors demonstrated that a composting process after ACoD can be applied, generating a stable and sanitised composted material that could be used as a soil improver.

This thesis provides novel information on raw microalgae and primary sludge ACoD, since this process was studied not only at laboratory-scale, but also at pilot-scale, which is a necessary step for future applications at industrial-scale. High stability and high substrates degradation, corresponding to high methane yield, were achieved co-digesting both substrates due to the synergy effects found between microorganisms and also due to the use of AnMBR technology, avoiding the application of costly pretreatments. This process is enclosed in a circular economy scenario in which resources (biogas, nutrients and water) are being recovered from urban wastewater.

Resum

El consum continu d'energia primària ha motivat a la comunitat científica a buscar tecnologies que requerisquen un menor consum de recursos i fonts alternatives d'energia renovable, que puguen substituir als combustibles fòssils. El cultiu de microalgues en combinació amb tecnologies anaeròbies va sorgir com una tecnologia prometedora i sostenible en l'àmbit del tractament d'aigües residuals. La valorització de la biomassa de microalgues per mitjà de la digestió anaeròbia (AD) permet la producció d'energia a partir de corrents de residus. Atès que la AD de microalgues presenta alguns inconvenients que obstaculitzen l'eficiència del procés, s'ha estudiat la co-digestió anaeròbia (ACoD) amb substrats rics en carboni, com a residus de paper o fangs, com a alternativa per a augmentar l'eficiència del procés anaerobi.

El present estudi consistix en l'avaluació a llarg termini de la ACoD de microalgues sense pretratar i fang primari. Per a això, la tecnologia de ACoD es va incloure en un marc ambientalment sostenible en el que la AD es va utilitzar en primer lloc per a l'eliminació de substàncies orgàniques de les aigües residuals decantades; les microalgues cultivades en una planta pilot de fotobioreactors de membrana es van utilitzar per a l'eliminació de nutrients de l'efluent de la AD; i la biomassa de microalgues i el fang primari es van co-digerir en un reactor de membrana anaerobi (AnMBR). Les microalgues i el fang primari es van co-digerir a escala de laboratori i pilot i es van avaluar diferents condicions operacionals tenint en compte els processos biològics, la filtració per membrana i la comunitat microbiana involucrada.

La ACoD de microalgues i fang primari es va estudiar primer a escala de laboratori, operant a diferents temps de retenció de sòlids (SRT), velocitat de càrrega orgànica (OLR) i temperatura. Els resultats van indicar que, quant major era el SRT i la OLR, major era la producció de metà, treballant en un AnMBR. Els resultats de laboratori també van mostrar que el AnMBR termòfil aconseguia una major producció de metà que el mesòfil. No obstant això, els balanços econòmics i energètics realitzats per a saber quins són les millors condicions d'operació per a escalar el procés de laboratori a escala pilot van mostrar que un SRT de 70 d i les condicions mesòfiles eren les recomanables per a operar la planta pilot de ACoD. Per tant, basant-se en els resultats previs, la planta pilot de ACoD es va operar durant un any a 70 d de SRT i 35 ºC. La planta pilot AnMBR va aconseguir una biodegradabilitat del 62% i va mostrar una alta estabilitat en termes de pH i àcids

grassos volàtils. Es va avaluar el procés de filtració, indicant que l'aplicació de la demanda específica de gas i un cicle de contrallavat per cada dos cicles de filtració va evitar la formació de fouling irreversible.

S'ha demostrat que la ACoD de microalgues augmenta la producció de metà en comparació amb la monodigestió de microalgues. L'addició d'un substrat fàcilment biodegradable com a fang primari a la biomassa microalgal va tindre un efecte sinèrgic en la AD, creant una comunitat microbiana adaptada capaç de degradar ambdós substrats per vies sintròfiques en les que microorganismes sintròfics com *Smithella* o *W5* degraden intermediaris (especialment propionat) i augmenten la producció de metà, principalment duta a terme per metanògens aceticlàstics com *Methanosaeta*. Els microorganismes hidrolítics i fermentadors que intervenen en la degradació de les proteïnes (per exemple, *Coprothermobacter*, *Fervidobacterium* o membres de la família Synergistaceae) i la degradació de la cel·lulosa (per exemple, *Defluviitoga* o *Thermogutta*) també van exercir un paper important en la degradació d'ambdós substrats.

En este treball es va avaluar també la possible recuperació de nutrients dels efluents de la ACoD (permeat i digestat). El nitrogen es va recuperar del permeat amb una eficiència del 99% utilitzant un contactor de membrana hidrofòbic de fibra buida de polipropilè. El fòsfor no es va recuperar del permeat ja que el 74% es va precipitar durant el procés de AD. Els experiments realitzats amb el digestat procedent de la ACoD en els reactors de compostatge Dewar, a escala laboratori, van demostrar que es pot aplicar un procés de compostatge després de la ACoD, generant un material compostat estable i higienitzat que podria utilitzar-se com a esmena orgànica.

Esta tesi proporciona informació nova sobre la ACoD de microalgues sense pretratar i fang primari, ja que este procés es va estudiar no sols a escala de laboratori, sinó també a escala pilot, la qual cosa constituïx un pas necessari per a futures aplicacions a escala industrial. Es va aconseguir una alta estabilitat i una elevada degradació dels substrats, (corresponent a un alt rendiment de metà) al co-digerir ambdós substrats degut als efectes de sinergia trobats entre els microorganismes i també pel fet que s'estava utilitzant la tecnologia AnMBR, evitant l'aplicació de costosos pretractaments. Este procés s'emmarca en un escenari d'economia circular en el que s'estan recuperant recursos (biogàs, nutrients i aigua) de les aigües residuals urbanes.

Resumen

El consumo continuo de energía primaria ha motivado a la comunidad científica a buscar tecnologías que requieran un menor consumo de recursos y fuentes alternativas de energía renovable, que puedan sustituir a los combustibles fósiles. El cultivo de microalgas en combinación con tecnologías anaerobias surgió como una tecnología prometedora y sostenible en el ámbito del tratamiento de aguas residuales. La valorización de la biomasa de microalgas mediante la digestión anaerobia (AD) permite la producción de energía a partir de corrientes de residuos. Dado que la AD de microalgas presenta algunos inconvenientes que obstaculizan la eficiencia del proceso, se ha estudiado la co-digestión anaerobia (ACoD) con sustratos ricos en carbono, como residuos de papel o fangos, como alternativa para aumentar la eficiencia del proceso anaerobio.

El presente estudio consiste en la evaluación a largo plazo de la ACoD de microalgas sin pretratar y fango primario. Para ello, la tecnología de ACoD se incluyó en un marco ambientalmente sostenible en el que la AD se utilizó en primer lugar para la eliminación de sustancias orgánicas de las aguas residuales decantadas; las microalgas cultivadas en una planta piloto de fotobiorreactores de membrana se utilizaron para la eliminación de nutrientes del efluente de la AD; y la biomasa microalgal y el fango primario se co-digirieron en un biorreactor de membrana anaerobio (AnMBR). Las microalgas y el fango primario se co-digirieron a escala de laboratorio y piloto y se evaluaron diferentes condiciones operacionales teniendo en cuenta los procesos biológicos, la filtración por membrana y la comunidad microbiana involucrada.

La ACoD de microalgas y fango primario se estudió primero a escala de laboratorio, operando a diferentes tiempos de retención de sólidos (SRT), velocidad de carga orgánica (OLR) y temperatura. Los resultados indicaron que, cuanto mayor era el SRT y la OLR, mayor era la producción de metano, trabajando en un AnMBR. Los resultados de laboratorio también mostraron que el AnMBR termófilo alcanzaba una mayor producción de metano que el mesófilo. No obstante, los balances económicos y energéticos realizados para saber cuáles son las mejores condiciones de operación para escalar el proceso de laboratorio a escala piloto mostraron que un SRT de 70 d y las condiciones mesófilas eran las recomendables para operar la planta piloto de ACoD. Por tanto, en base a los resultados anteriores, la planta piloto de ACoD se operó durante un año a 70 d SRT y 35 ºC. La planta piloto AnMBR alcanzó una biodegradabilidad del 62% y mostró una alta

estabilidad en términos de pH y ácidos grasos volátiles. Se evaluó el proceso de filtración, indicando que la aplicación de la demanda específica de gas y un ciclo de contralavado por cada dos ciclos de filtración evitó la formación de fouling irreversible.

Se ha demostrado que la ACoD de microalgas aumenta la producción de metano en comparación con la monodigestión de microalgas. La adición de un sustrato fácilmente biodegradable como fango primario a la biomasa microalgal tuvo un efecto sinérgico en la AD, creando una comunidad microbiana adaptada capaz de degradar ambos sustratos por vías sintróficas en las que microorganismos sintróficos como *Smithella* o *W5* degradan intermediarios (especialmente propionato) y aumentan la producción de metano, principalmente llevada a cabo por metanógenos aceticlásticos como *Methanosaeta*. Los microorganismos hidrolíticos y fermentadores que intervienen en la degradación de las proteínas (por ejemplo, *Coprothermobacter*, *Fervidobacterium* o miembros de la familia Synergistaceae) y la degradación de la celulosa (por ejemplo, *Defluviitoga* o *Thermogutta*) también desempeñaron un papel importante en la degradación de ambos sustratos.

En este trabajo se evaluó también la posible recuperación de nutrientes de los efluentes de la ACoD (permeado y digestato). El nitrógeno se recuperó del permeado con una eficiencia del 99% utilizando un contactor de membrana hidrofóbica de fibra hueca de polipropileno. El fósforo no se recuperó del permeado ya que el 74% se precipitó durante el proceso de AD. Los experimentos realizados con el digestato procedente de la ACoD en los reactores de compostaje Dewar, a escala laboratorio, demostraron que se puede aplicar un proceso de compostaje después de la ACoD, generando un material compostado estable e higienizado que podría utilizarse como enmienda orgánica.

Esta tesis proporciona información novedosa sobre las ACoD de microalgas crudas y fango primario, ya que este proceso se estudió no solo a escala de laboratorio, sino también a escala piloto, lo que constituye un paso necesario para futuras aplicaciones a escala industrial. Se logró una alta estabilidad y una elevada degradación de los sustratos, (correspondiente a un alto rendimiento de metano) al co-digerir ambos sustratos debido a los efectos de sinergia encontrados entre los microorganismos y también debido a que se estaba utilizando la tecnología AnMBR, evitando la aplicación de costosos pretratamientos. Este proceso se enmarca en un escenario de economía circular en el que se están recuperando recursos (biogás, nutrientes y agua) de las aguas residuales urbanas.

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Introduction

1. INTRODUCTION

1.1. NEW TECHNOLOGIES IN WASTEWATER TREATMENT

The continual growth in the world's population has brought about an increase in the generation of municipal/industrial waste and the consumption of fossil fuels. Then, legislation in most countries is driving research into new wastewater treatment plant (WWTP) configurations, establishing higher restrictions for effluents and sludge's disposal and imposing the necessity for WWTPs of being energy self-sufficient or even generating a positive net energy balance. Thus, a paradigm shift from the traditional urban wastewater treatment (WT) to a sustainable system is required.

A large number of organizations (governmental and non-governmental) have recognised the potential from a social, economic and environmental point of view of resources present in wastewater (Mihelcic et al., 2017). WT technologies are no longer considered pollution removal systems but rather systems for resources recovery. Traditional WWTP are being replaced by new wastewater resource recovery facilities (WRRF), in which water, energy and nutrients are recovered from waste. However, guiding the current WT infrastructures into a new generation of WRRF would require a huge investment in development and implementation of new technologies and processes, using innovative software, advanced devices and smart sensors.

Several processes have been studied in the last decade to improve resource recovery from wastewater. WRRFs may include in their layout an anaerobic system that allows carbon recycling, generating bioenergy from different wastes (Kougias and Angelidaki, 2018). High rate activated sludge (HRAS) has also been reported as an acceptable technology for carbon redirection towards energy as biogas in WWTP, with a lower energy demand, and thus saving costs (Sancho et al., 2019). Membrane-based technologies, such as forward osmosis, electrodyalisis, biolectrochemical systems (upgraded by integrating membrane technology) or anaerobic membrane bioreactors (AnMBRs) have been reported to improve energy and nutrients recovery efficiencies (Robles et al., 2020; Volpin et al., 2019; Yang et al., 2019). Another technological innovation is the introduction of photosynthetic-based technologies for WT. Microalgae-based WT has been found as a feasible alternative for nutrients recovery (González-Camejo et al., 2017) and, linked to the posterior anaerobic digestion (AD) of the produced algae biomass

(Greses, 2017), has been observed to increase methane production. These technologies, among others, are being studied at laboratory and pilot-scale, but still present hindrances that must be solved for full-scale implementation. Research needs to be addressed in this direction, looking into new technologies that could increase energy and nutrients recovery efficiencies, and therefore could save costs.

1.2. ANAEROBIC DIGESTION OF ORGANIC MATTER

The AD process is extensively being applied at industrial-scale for organic residues valorisation. According to EBA (2019), 18202 biogas plants and 660 biomethane plants are in operation in Europe. AD is the organic matter degradation that occurs in absence of oxygen. It is a microbial mediated process in which carbon is turned into CO_2 and CH_4, after a series of reactions involving oxidations and reductions. Then, this process is used for carbon recycling in different environments such as animal' intestines, manures, sediments or wetlands (Kougias and Angelidaki, 2018). But AD is also used in many countries for waste conversion to biogas, since biogas is the final product of this process and it can be converted to energy.

1.2.1. Stages in anaerobic digestion process and microorganisms involved

AD is a sequence of consecutive and simultaneous phases in which products from each stage are used as substrates in the subsequent stage, catalysed by a wide range of microorganisms acting in synergy. AD process could be divided into four key steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis (see Figure 1.1).

Hydrolysis is the first step of anaerobic degradation, in which complex organic polymers such as proteins, lipids and carbohydrates are broken down into simpler molecular compounds such as sugars, amino acids, long fatty acids and alcohols by the action of hydrolytic extracellular enzymes from fermentative bacteria and fungi (Kazda et al., 2014). Hydrolytic bacteria can be found in phyla: Firmicutes, Bacteroidetes, Chloroflexi, Thermotogae and Proteobacteria, Firmicutes and Bacteroidetes being the most abundant phyla and *Clostridium* one of the most abundant genera (Nguyen et al., 2018). Subsequently, the simpler organics released from carbohydrates and proteins decomposition are available for fermentative bacteria (acidogens) in order to form volatile fatty acids (VFA), which are short-chain fatty acids such as butyric, propionic or acetic acid; alcohols such as ethanol and lactate; and other methane precursors (CO_2 and

hydrogen). The final fermentation product and its proportion depends on the microbial community established and on their thermodynamics. Acidogenesis is carried out by bacteria related to phyla Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria, the most abundant genera being: *Clostridium*, *Pseudomonas*, *Desulfovibrio*, *Bacillus*, *Micrococcus* and *Flavobacterium*.

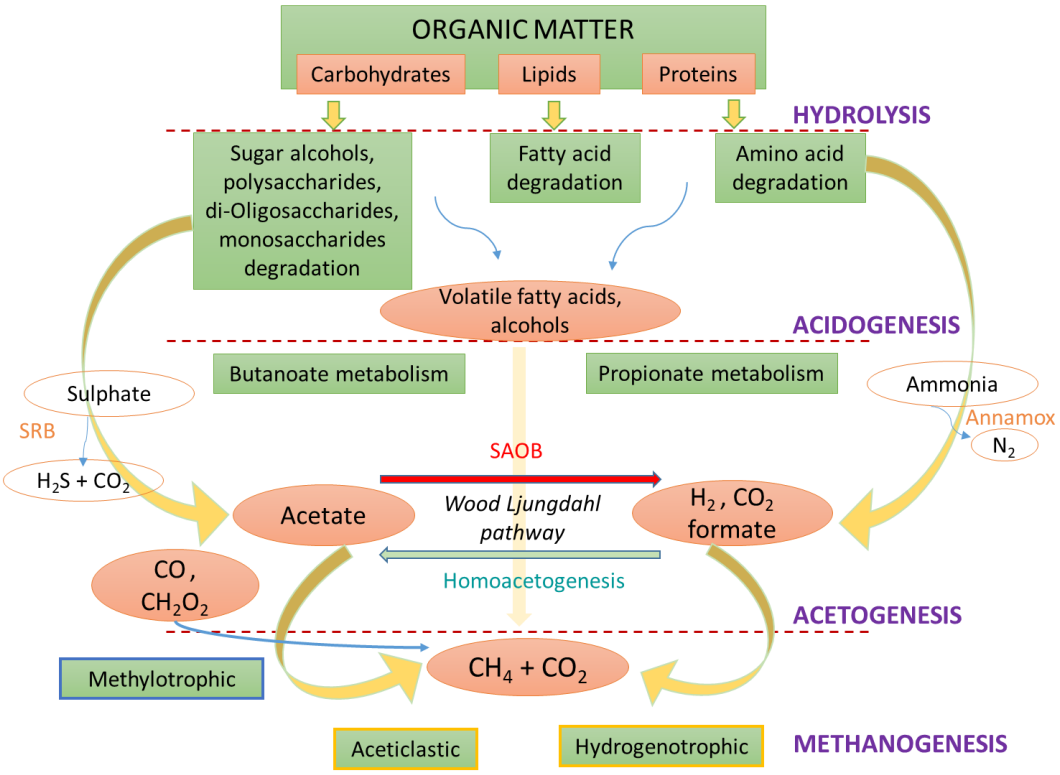

Figure 1.1. Organic matter anaerobic degradation. Source: adapted from Kougias and Angelidaki (2018).

VFA with more than two carbons and alcohols are oxidised to more acetic acid, hydrogen and carbon dioxide during acetogenesis. In this acetogenesis step, syntrophic acetate-oxidising bacteria (SAOB) oxidise acetate to CO_2 and H_2 while homoacetogens reduce CO_2 and H_2 to acetate through a pathway named as Wood Ljungdahl (WL) pathway or reductive Acetyl-CoA pathway. Westerholm et al. (2016) reported that under specific AD conditions in terms of temperature, ammonia (NH_3) concentration, or substrate feed, among others, thermodynamic equilibrium between acetate, H_2 and CO_2, would decide on the metabolic pathway shift between homoacetogenesis or syntrophic acetate oxidation. Acetogenesis requires very low hydrogen partial pressure to favour the reactions thermodynamics (Table 1.1). Hence, SAOB could cause toxic effects on the

process, because of the high hydrogen pressure accumulation on the system. A syntrophic relation with hydrogen consuming microorganisms such as methanogens or sulphate reducing bacteria (SRB) is needed to avoid a process inhibition by hydrogen. Hydrogenotrophic methanogens keep the hydrogen pressure low, allowing fermentative bacteria to oxidise the organic compounds (Barua and Dhar, 2017). SRB also keep the low H_2 pressure, since these microorganisms consume H_2 to reduce sulphate to sulphide. These bacteria run the WL pathway in its oxidation direction (Ragsdale and Pierce, 2008), coupling the endergonic oxidation of acetate to CO_2 and H_2 to the exergonic reduction of sulphate to sulphide (Table 1.1).

Long-chain fatty acids and glycerol obtained from lipids hydrolysis are further degraded to acetate and hydrogen through β-oxidation by syntrophic acetogenic bacteria. In substrates with high lipid content, this is the slowest step and controls the AD kinetics (Ma et al., 2015). The thermodynamic feasibility of the β-oxidation pathway also relies on low partial pressure of hydrogen, needing again a syntrophic association with hydrogen consuming microorganisms.

Propionate degradation is also key during AD as biogas production can be severely destabilised by propionate accumulation (Wang et al., 2006). Propionate degradation is carried out by propionate oxidising bacteria (POB) in a syntrophic relation with hydrogen consuming microorganisms since this degradation is a thermodynamically unfavourable reaction (Table 1.1) that also requires low H_2 partial pressure.

An important number of acetogens belong to genera *Syntrophomonas* and *Syntrophobacter*, but other non-syntrophic acetogens such as *Clostridium aceticum* are also abundant.

Finally, methanogenesis is the production of methane from H_2 and CO_2 (hydrogenotrophic methanogenesis), acetate (aceticlastic methanogenesis) or methylated compounds like methanol or methylamine (methylotrophic methanogenesis) (Table 1.1) carried out mainly by archaeal methanogens. Thermodynamic conditions play a key role in methane formation. Even reactions for methane formation are energetically favourable reactions, with negative Gibbs free energy (Table 1.1), methanogenesis is a critical step on AD since methanogens are quite sensitive to changes in operating and environmental conditions such as VFA accumulation, high NH_3 concentration, changes in temperature,

etc. Hence, appropriate environmental conditions should be provided to perform acetogenesis and methanogenesis, simultaneously. Methanogenic archaea are related to five orders: Methanobacteriales, Methanosarcinales, Methanococcales, Methanomicrobiales and Methanopyrales. Among them, *Methanosaeta*, a strict aceticlastic methanogen and *Methanosarcina*, which has versatile metabolic pathways, are abundant genera (Nguyen et al., 2018). Hydrogenotrophic methanogens such as *Methanoculleus* or *Methanospirillum* and methylotrophic methanogens such as *Methanolobus*, are also common in anaerobic digesters.

Table 1.1. Gibbs-free energy changes for different pathways in anaerobic digestion process.

Reaction and stoichiometry	**ΔG^0**	**Reference**
Butyrate oxidation	+48.1	Kim et al. (2018)
$CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2$		
Propionate oxidation	+76.1	Kim et al. (2018)
$CH_3CH_2COO^- + 3\ H_2O \rightarrow CH_3COO^- + H^+ + HCO_3^- + 3H_2$		
Acetate formation	-104.5	Angelidaki et al. (2018)
$2CO_2 + 4H_2 \rightarrow CH_3COO^- + H^+ + 2H_2O$		
Hydrogenotrophic methanogenesis	-136	Sousa et al. (2007)
$HCO_3^- + 4H_2 \rightarrow CH_4 + 3H_2O$		
Aceticlastic methanogenesis	-31	Sousa et al. (2007)
$CH_3COO^- + H_2O \rightarrow HCO_3^- + CH_4$		
Methane formation from methanol	-112.5	Thauer et al. (2008)
$CH_3OH + H_2 \rightarrow CH_4 + H_2O$		
Reduction of sulphate coupled to acetate oxidation	-57	Ragsdale and Pierce (2008)
$SO_4^{2-} + CH_3COO^- + 2H^+ \rightarrow HS^- + 2CO_2 + 2H_2O$		

ΔG^0: standard Gibbs-free energy at 25 ºC expressed in kJ·mol^{-1}.

1.2.2. Anaerobic digestion technologies

Organic matter from waste can be converted into biogas in a digester through AD process. Digesters are designed to provide optimal conditions for microorganisms (pH, temperature, mixing, etc.) and can be classified according to temperature of operation (psychrophilic, mesophilic or thermophilic), feeding conditions (i.e. batch, semi-continuous, continuous) or number of stages (mono-stage or two-stages). The original AD technology (the covered lagoon) is rarely used nowadays due to environmental and efficiency considerations. Nevertheless, different reactor configurations have been developed in the last decades and are currently being used for the anaerobic treatment of industrial wastes and wastewaters. Among them, the most common types are: completely mixed anaerobic reactors, anaerobic filters (AF), fluidized and expanded bed reactors, upflow anaerobic sludge blanket (UASB) reactors and AnMBRs.

The completely mixed anaerobic reactors are the basic AD systems in which hydraulic retention time (HRT) and solids retention time (SRT) are equal. Continuous-stirred tank reactors (CSTRs) are mono-stage systems (Figure 1.2), frequently used in AD systems, especially in research field, because of their simplicity in design and operation, efficient and uniform stirring and correct maintenance of pH and temperature (Usack et al., 2012). The disadvantage of this bioreactor application is the large volume required and the rapid washout of the biomass when the dilution rate exceeds the maximum biomass growth rate.

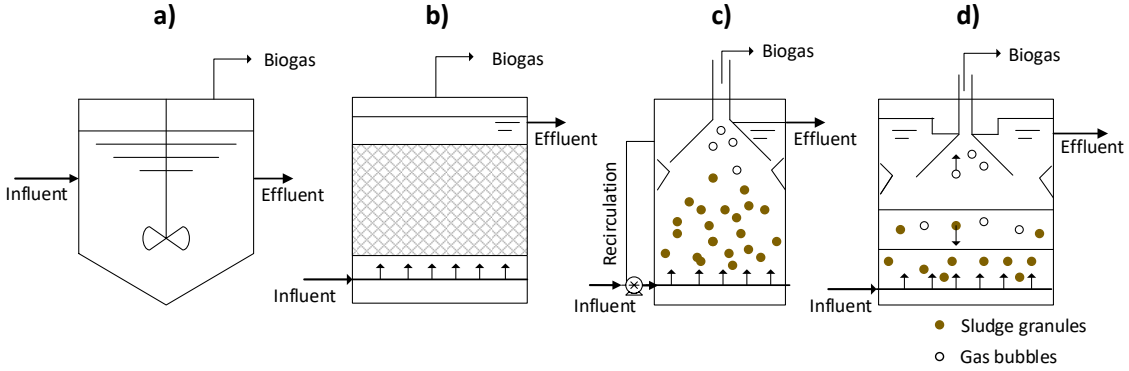

Figure 1.2. Different anaerobic reactors types: continuous stirred tank reactor (a), anaerobic filter (b), anaerobic fluidized or expanded bed (c) and upflow anaerobic sludge blanket (d).

AF (Figure 1.2b) were the first anaerobic systems that removed the necessity for solids separation while providing a high SRT:HRT ratio. The support media are made from materials such as plastics, granular activated carbon, ceramics, minerals, glass or sand. These reactors are suitable for treating high-strength soluble wastewater and are mainly used for treating food and distillery effluents. However, the deterioration of the bed structure because of the accumulation of non-biodegradable solids as well as the high cost of packing materials are important limitations for this technology that make them unsuitable for high-solids feedstock streams (Anderson et al., 2003). A more recent technology is the anaerobic fluidized bed reactor (AFBR) (Figure 1.2c). This reactor comprises small media, such as granular activated carbon or sand, to which bacteria are attached. The use of small porous fluidizing media enables the reactor to retain high biomass concentrations and thereby operate at significantly reduced HRT. However, the high energy cost and the difficulty of finding strongly attached biofilm, led to a modification in the reactor to: expanded granular sludge bed (EGSB). The upflow velocity in EGSB is not as high as in the AFBR, which results in partial bed fluidization. One of the most important risks associated with the AFBR and EGSB reactors is the loss of biomass particles from the reactor as a consequence of sudden changes in particle density, flow rate or gas production. Besides, difficulties have been reported in controlling the density of the flocks and particle size since biomass growth on the particles is variable. The problem associated with packed bed AF and AFBR and EGSB reactors has led to the development of unpacked reactors that still incorporate an immobilized form of particulate biomass in which wastewater flows through a sludge blanket consisting of a granular sludge bed. One of the most successful configurations implemented in AD systems was the UASB reactor, by Lettinga et al. (1980) (Figure 1.2d), which eliminates the packing material cost, offering good sedimentation and high biomass concentrations. However, this system still presents some disadvantages such as the generation of a high nutrient effluent with presence of pathogens, requiring a subsequent step for nutrients and pathogens removal (Chong et al., 2012). Other problems associated with UASB reactors are related to flotation and disintegration of granular sludge (Li et al., 2008). Moreover, UASB reactors are a well-established technology for high-strength wastewaters but present an important limitation when treating high suspended solids wastewater since granulation could be inhibited resulting in a low performance. Then, even the described systems (AF, AFBR, EGSB and UASB) sorted

out one of the main barriers for anaerobic degradation, as high SRTs are required for anaerobic microorganisms to growth, they still present the drawback that are not suitable technologies for high-solids influents.

A continuous two-stages AD system consisting of an acidogenic step followed by a methanogenic step could be a feasible solution to the abovementioned problems. One common way of presenting these two-stages systems is the combination of an acidogenic CSTR followed by a methanogenic UASB (Asato et al., 2019). The incorporation of an UASB reactor in this system prevents microorganisms from being washout due to the decoupling of HRT from SRT, using a dense granular sludge. Then, combining an UASB with a CSTR could overcome the high-solid feedstock limitation. Nonetheless, other disadvantages inherent to both systems are still present.

1.2.2.1. Anaerobic membrane bioreactors (AnMBRs)

AnMBRs, which combine the anaerobic process and membrane technology, have arisen as a promising technology for WT in preference to conventional aerobic treatments for their several advantages (Giménez et al., 2011). These reactors are attracting remarkable interest in both industrial and scientific sectors. The AnMBR concept was first described by Grethlein et al. (1978) while the first AnMBR was commercially available in the early 1980s for high-strength whey processing WT. AnMBR systems have been used for industrial effluents treatments at full-scale (Dereli et al., 2012) while have been used for low-strength wastewater at pilot-scale (Giménez et al., 2011, Shin and Bae, 2018). Two principal AnMBR configurations can be established depending on how the membrane is connected to the reactor: immersed or sidestream (Robles et al., 2018), as seen in Figure 1.3. The immersed membrane configuration is generally used for low-strength WT while the sidestream membrane configuration is usually applied to high-strength WT.

AnMBR technology offers several advantages: (i) allows decoupling of HRT from SRT, which avoids microorganisms from being washout due to biomass retention in the membrane; (ii) presents high efficiency in terms of organic matter and suspended solids removal, offering a high quality effluent in which microbiological contamination has also been eliminated (bacteria, parasites, microalgae), (iii) allows ambient-temperature AD operation by increasing the SRT even at cold climates (iv) leads to a lower amount of biosolids for disposal because of the low growth of anaerobic biomass, and these biosolids are more stabilised due to the high SRT, (v) allows a posterior nutrient recovery since

they are not destroyed during AD and (vi) removes suspended organic matter, making a further tertiary treatment easier (Robles et al., 2018). Nevertheless, when operating a membrane-based system there are some issues that should be considered: (i) a post-treatment step may be needed due to the high nutrient concentration in the effluent (ii) methane concentration dissolved in the effluent raises as the operating temperature decreases, being an important drawback for operation in cold/mild regions (Crone et al., 2016); this dissolved methane need to be captured to avoid its stripping to the atmosphere and (iii) membrane fouling and cleaning is a critical factor for membrane operation.

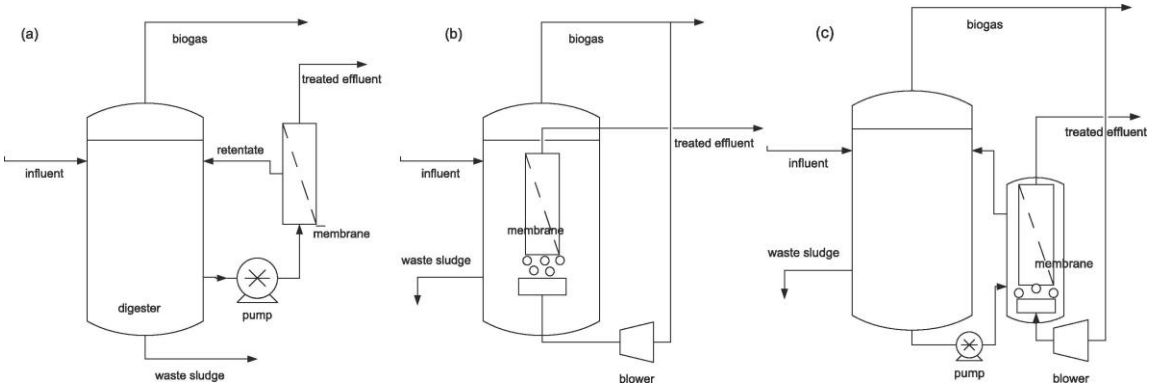

Figure 1.3. AnMBR configurations: sidestream membrane bioreactor (a), immersed membrane bioreactor (b), combination of both "a" and "b" configurations (c). Source: Robles et al., 2018.

- **Membrane fouling limitation**

The major concern associated with AnMBR technology is membrane fouling, which reduces permeate flux through the membrane due to the accumulation of organic and inorganic compounds in the membrane pores or surface, increasing energy consumption (Gong et al., 2019). Fouling is especially troubling when filtrating substrates with high organic matter and suspended solids such as microalgae biomass (Wang et al., 2019). The accumulation of soluble microbial products (SMPs) such as proteins and polysaccharides inside the reactor is reported to be key in membrane fouling since it could lead to cake layer formation and/or membrane pore blocking (Aslam et al., 2018). These two problems have been identified as the main fouling mechanisms in AnMBR systems (Dereli et al., 2012). Physical cleaning could be a solution for cake layer removal but frequently fouling cannot be physically removed to an acceptance level and a chemical cleaning is needed, either in situ (clean in place (CIP)) or ex situ. However, it has to be considered that

chemical cleaning is normally carried out using chemical agents that shorten membrane lifetime. Occasionally, neither physical nor chemical cleaning is enough because irrecoverable fouling has been formed, that is not removed by cleanings. Then, the most adequate strategy for membrane operation is to optimise process to avoid fouling formation. Biogas sparging is widely applied in AnMBR to control membrane fouling. Robles et al. (2013) reported that gas sparging intensity and back-flush frequency are the physical variables that affect membrane-cleaning most. They established a suitable physical cleaning schedule operating the membranes under subcritical transmembrane pressure conditions, observing no significant irreversible/irrecoverable fouling problems when treating urban wastewater, even when no chemical cleaning was conducted. Lei et al. (2018) found that the combination of recycling biogas to the membranes with backwashing could sufficiently control internal fouling, although pore blocking partially led to irreversible fouling when treating municipal wastewater.

1.3. ANAEROBIC EFFLUENT POST-TREATMENT

Apart from biogas, large quantities of digestate are produced during AD, which still presents a high nutrient concentration (nitrogen (N) and phosphorus (P)) as a result of the organic matter mineralization during AD. Digestate is generally mechanically separated into liquid and solid fractions, or, in the case of technologies integrating membranes such as AnMBRs, liquid-solid separation is occurring during AD process, obtaining two effluent streams: permeate and solid digested sludge. The liquid fraction contains a large part of N and P, and these concentrations commonly exceed the legal requirement for a direct discharge to water bodies, then, a post-treatment step is needed for nutrient removal or recovery. Traditional WWTPs used to remove N through a biological nitrification/denitrification step and P through an enhanced biological P removal or chemical precipitation. But, the challenge for WWTPs plants nowadays is to achieve optimal recovery and recycling of nutrients from the digestate or liquid effluent streams in a sustainable way. Different techniques have been reviewed in literature (Batstone et al., 2015, Robles et al., 2020, Vaneeckhaute et al., 2017) for nutrient removal or recovery, depending on the input waste stream characteristics: air stripping, crystallization-based systems, microalgae production, annamox process for ammonia oxidation via nitrite, fertirrigation, membrane-based systems, ion exchange, microbial fuel cells (MFC), microbial electrolysis cells (MEC) and other physical and biological technologies such as

incineration, composting or absorption. Among all of these techniques, microalgae production has been thoroughly studied due to microalgae ability to assimilate nutrients from wastewater (Judd et al., 2015).

1.3.1. Microalgae

Researchers have globally emphasised on the advantages of using microalgae for different innovative applications such as production of high-value compounds, production of fertilisers for agricultural industry, carbon mitigation, biofuel production and production of human or animal's food compounds. Garrido-Cárdenas et al. (2018) found an increasing trend on microalgae field research during the last decades, 2700 being the number of research articles published during year 2017.

These microorganisms show metabolic flexibility; although they are generally photoautotrophic organisms, their metabolism can also be mixotrophic, photoheterotrophic or heterotrophic. Photoautotrophic microalgae have a photosynthetic metabolism and use light (energy source) and inorganic carbon (CO_2 and HCO_3^-) to grow, whereas heterotrophic microalgae are non-photosynthetic microorganisms that require an external source of organic compounds to survive. Some microalgae are mixotrophic and they can present both photoautotrophic or heterotrophic metabolism, depending on light conditions and substrate availability. Apart from carbon source and light, microalgae also require nutrients to grow, such as ammonium and phosphate, which can be obtained from a nutrient-rich wastewater stream (Acién et al., 2018). Then, algae-based wastewater bioremediation appears as a promising option for WRRF. Microalgae could also remove other pollutants from wastewater such as organic pollutants, heavy metals, emerging contaminants and other drugs (Vo et al., 2019).

Microalgae have been reported to grow assimilating nutrients from wastewaters with different characteristics: industrial wastewater (Umamaheswari and Shanthakumar, 2016; Wang et al., 2016), agricultural wastewater (Khalid et al., 2019), agro-industrial wastewater (Hernández et al., 2013; Posadas et al., 2014) and urban wastewater (Acién et al., 2016; González-Camejo et al., 2020a). According to the effluent composition in terms of turbidity, chemical oxygen demand (COD), N, P and total suspended solids (TSS) (Acién et al., 2018), process should be adequately designed and operated.

Microalgae technology could be applied at different points of the WT process flow: (i) raw wastewater (Mennaa et al., 2015; Wang et al., 2010), (ii) primary or secondary treatment effluents (Gao et al., 2018; Shurair et al., 2017; Wang et al., 2010) (iii) centrates (Caporgno et al., 2015; Min et al., 2011; Ren et al., 2017; Wang et al., 2010) and (iv) AD (such as AnMBR or UASB treatment) effluents (González-Camejo et al., 2020a; Jesus et al., 2019; Viruela et al., 2017). The effluent from an AnMBR pilot plant treating real sewage has been demonstrated to be feasible for microalgae cultivation (González-Camejo et al., 2020a; Seco et al., 2018). AnMBR effluents present low organic matter content due to the high yields in organics and solids removal, and high nutrients concentration due to the organic matter mineralisation (Giménez et al., 2011). González-Camejo et al. (2020b) operated a membrane photobioreactor pilot plant (MPBR) continuously outdoors during three years. This MPBR plant treated an AnMBR effluent with N and P concentrations of 53 $mgN \cdot L^{-1}$ and 6 $mgP \cdot L^{-1}$, respectively, obtaining high nutrients removal efficiency: N and P concentrations were below the legal discharge limits (i.e., 15 $mgN \cdot L^{-1}$ and 2 $mgP \cdot L^{-1}$ according to European Directive 91/271/CEE for a 10,000-100,000-PE WWTP). This microalgae biomass could be later digested in a next AD step, as will be explained in Section 1.4.

1.3.2. Composting

The nutrient-rich digestate obtained in AD processes (Nag et al., 2019; Nkoa, 2013; Seco et al., 2018) is an organic matrix with agronomic properties, being a potential fertiliser and soil improver. Indeed, Tambone et al. (2010) studied eight digestates coming from municipal WWTP, finding that AD digestate had properties similar to compost as soil improver and could be a good nutrient source due to its high available N, P and potassium (K) content. However, digestate direct land application presents some limitations: i) digestate produced during the year has to be stored since it cannot be used immediately on farm lands and this storage can lead to gas emissions (i.e. CO_2, CH_4, NH_3 and nitrite (NO_2)) to the atmosphere; ii) a high amount of digestate is generated in AD plants that is excessive for local farms supply and implies transporting the excess digestate to other regions, which is not a solution due to the high cost; iii) in some cases (especially when digesting substrates with high volatile solids (VS) content such as microalgae biomass) digestate still has undigested VS, that will be converted into NH_3 and CH_4 during land uses or storage and (iv) digestate may contains pathogens, heavy metals, pesticides or

other microcontaminants that are environmental pollutants (Fuchs and Drosg, 2013; Monlau et al., 2015). These limitations make a posterior process (e.g. composting, drying, or evaporation) required to generate a stable organic improver.

Composting is the biological decomposition of organic waste that takes place under controlled aerobic conditions and involves mesophilic and thermophilic microorganisms. In this process, the biodegradable organic substrates are transformed into a stabilised material, a humus-like product free of pathogens and plant seeds, ready to be used for agricultural purposes.

Composting is divided in two phases, decomposition and maturation phases, in which mesophilic and thermophilic microorganisms are the most abundant. The decomposition phase, in which there is a high abundance of substrates and the highest degradation of the organic matter occurs, is divided in two stages, the mesophilic and thermophilic ones. In the mesophilic stage, large amount of heat energy is generated, rising the temperature of the compost pile, up to 45 ºC. During the thermophilic stage, the compost pile reaches temperatures up to 70 ºC, taking place the pathogen removal. The maturation phase is also divided in two stages: the cooling stage, in which the temperature drops until ambient values and the stabilisation stage, in which the biological activity is the lowest and it is characterised for the presence of upper organisms as mites or collembolans.

Composting process depends on several environmental factors such as pH, aeration, moisture content or temperature but also depends on sludge characteristics such as nutrients content, particle size or carbon/nitrogen (C/N) ratio (Nikaeen et al., 2015). Although composting process involves different kind of microorganisms that work at different pH, it is well established that the optimum pH for composting should be between 6.5 and 8.5 (Silva and Naik, 2007), depending on the stage of the process. Since aerobic conditions are required, an adequate aeration is necessary for an efficient process. Aeration allows water to be removed from wet substrates and temperature control since heat is being generated while organic decomposition occurs. Moisture content is also an important factor for bacteria to assimilate the necessary nutrients. There are several studies indicating different optimum moisture content, but, generally, a moisture percentage between 50 and 60% is the recommendable content for composting process (Kim et al., 2016). While a low moisture content below 50% limits microbial activity (Liang et al., 2003), an excessive water content could lead to stench since water avoids

air passing through the compost, creating anaerobic conditions. Temperature control is important for maximising the organic matter decomposition and assuring pathogens removal but it is also important for maintaining the correct moisture content.

C/N ratio is a key parameter for composting process and compost quality. An optimum C/N ratio between 25-30 has been reported for composting process as well as for AD process (Huang et al., 2004; Puyuelo et al., 2011). Substrates do not normally present this optimum C/N ratio, especially substrates with high N content such as microalgae or sludge (Solé-Bundó et al., 2019; Ullah et al., 2015). Then, due to their low carbon and high moisture content, sludges need to be mixed with dry materials, called bulking agents (BAs) for composting. Agricultural wastes, sawdust, tree branches, plant residues, among others, can be used as BAs.

1.4. ANAEROBIC DIGESTION OF MICROALGAE

Growing microalgae in wastewater has other benefits apart from removing nutrients from wastewater streams. Microalgae biomass can assimilate large quantities of inorganic and organic carbon, which will otherwise be emitted as CO_2 in the atmosphere, converting this carbon source into biomass, which can then be processed downstream for biofuel production.

Particularly, oleaginous microalgae have been investigated for their capacity to accumulate oil exceeding 50% of dry weight in triacylglycerol form. Studies published on biofuel production focused on some algae including Chlorophyta phylum (green algae) such as *Chlorella*, *Scenedesmus*, *Monoraphidium*, *Chlamydomonas*, *Neochloris* or *Nannochloris*; Heterokontophyta phylum such as *Nannochloropsis;* or Haptophyta phylum such as *Isochrysis* or *Pavlova* (Klassen et al., 2016; Larkum et al., 2012).

The concept of using algae as a fuel was first proposed by Meier (1955) for methane gas production from carbohydrate fractional cells and this idea was developed some years later by Golueke et al. (1957), who studied *Scenedesmus* and *Chlorella* AD and compared it with raw sewage sludge digestion at laboratory scale. Since then, there has been a growing research interest for microalgae in bioenergy field due to the algae high biomass yield and the cultivation system: microalgae do not compete for land or fresh water with terrestrial crops appearing as a promising feedstock for the ideal third generation biofuel.

Microalgae composition affects the theoretical biochemical methane potential (BMP) of algae. The carbohydrates, lipids and proteins composition varies widely depending on microalgae specie, season and environmental conditions, which is challenging for reproducibility in biogas generation at full-scale (Milledge et al., 2019). The carbohydrate, lipid and protein composition of various microalgae species is shown in Table 1.2.

Table 1.2. Protein, carbohydrate and lipid content of various microalgae % dry weight (Roy et al., 2014)

Microalgae	**Proteins**	**Carbohydrates**	**Lipids**
Spirulina platensis	50-65	8-14	4-9
Chlorella sp.	51-58	12-17	14-22
Scenedesmus sp.	50-56	10-52	12-14
Dunaliella sp.	49-57	4-32	6-8
Synechococcus sp.	63	15	11
Euglena sp.	39-61	14-18	14-20
Prymnesium sp.	28-45	25-33	22-38
Anabaena sp.	48	25-30	4-7
Chlamydomonas sp.	43-56	2.9-17	14-22
Spirulina maxima	60-71	13-16	6-7
Spirogyra	6-20	33-64	11-21
Tetraselmis	52	15	16-45
Pavlova	24-29	6-9	9-14
Porphyridum sp.	28-39	50-57	-

Generally, the microalgae biomass BMP is higher than crop plants BMP such as corn or maize (Murphy et al., 2015; Weiland, 2010), because of the higher amount of protein and lipids content in microalgae (Klassen et al., 2016). However, microalgae AD does not often achieve the theoretical BMP (TBMP). Table 1.3 shows the difference between TBMP and experimental BMP for different microalgae species studied for AD processes. The TBMP values were calculated as described by Heaven et al. (2011) based on protein,

lipids and carbohydrates content. In those studies, in which microalgae composition was not published, a value of 549 $mLCH_4 \cdot gVS^{-1}$ was assumed according to Klassen et al. (2016). Different results can be found for the same microalgae species, depending on several factors such as the temperature of operation or the microalgae cultivation method. Nonetheless, as seen in Table 1.3, only a few experiments have reached further than 60% the theoretical BMP, but, it should be considered that between 5 and 15% of organic matter is used for new microbial biomass generation (Raposo et al., 2011). Then those experiments in which 70% of biomass conversion into methane is achieved are near to the TBMP. All experiments shown in Table 1.3 are based on batch experiments. Most of these experiments have been later carried out in continuous reactors, achieving lower methane yields. The low yields could be attributed to the limitations to biodegradation that algae present: a cell wall highly resistant towards degradation and a low C/N ratio that can lead to an excess of ammonia in inhibiting concentrations for AD process. These two factors could result in low AD performances and are further discussed in the next sections (1.4.1 and 1.4.2).

Table 1.3. Biomethane potential (BMP) values for different untreated microalgae species. The theoretical BMP (TBMP) values were calculated as described by Heaven et al. (2011), assuming values of 415 mL, 446 mL and 1014 $mLCH_4 \cdot gVS^{-1}$ for carbohydrates, proteins or lipids, respectively.

Microalgae	**TBMP** ($mLCH_4 \cdot gVS^{-1}$)	**BMP**	**%BMP of TBMP**	**Reference**
Chlorella vulgaris	542	293	54	Mahdy et al., 2014a
Chlorella vulgaris	489	328	67	Mahdy et al., 2014b
Chlorella vulgaris	549	229	42	Park et al., 2013
Chlorella vulgaris	516	318	62	Lü et al., 2013
Chlorella vulgaris	502	250	50	Mendez et al., 2013
Chlorella vulgaris	539	339	63	He et al., 2016
Chlorella sorokiniana	549	98	18	Polakovicova et al., 2012
Parachlorella kessleri	549	240	44	Klassen et al., 2015

(Continues in next page)

Microalgae	**TBMP**	**BMP**	**%BMP of TBMP**	**Reference**
	($mLCH_4 \cdot gVS^{-1}$)			
Chlorella sp. 70% *Scenedesmus* sp. 30%	549	336	51	Cho et al., 2013
Chlorella, Stigeoclonium	542	90	17	Solé-Bundó et al., 2018
Chlorella, Scenedesmus	549	319	58	Olsson et al., 2014
Scenedesmus sp.	549	127	23	González-Fernández et al., 2012a
Scenedesmus sp.	572	222	39	Mahdy et al., 2015a
Scenedesmus sp.	549	54	10	Inglesby et al., 2015
Scenedesmus obliquus	549	210	38	Zamalloa et al., 2012
Scenedesmus obliquus	549	213	39	Klassen et al., 2015
Chlamydomonas reinhardtii	511	405	79	Mahdy et al., 2014a
Chlamydomonas reinhardtii	549	290	53	Klassen et al., 2015
Chlamydomonas 40% *Scenedesmus* 40%	549	272	50	Alzate et al., 2012
Nannochloropsis salina	637	200	31	Schwede et al., 2013
Nannochloropsis oculata	549	206	38	Marsolek et al., 2014
Arthrospira platensis	486	370	76	Aramrueang et al. 2016
Spirulina platensis	549	280	51	Zamalloa et al., 2012
Monoraphidium, Stigeoclonium sp. *Nitzschia* sp.	549	148	27	Passos et al., 2014a

1.4.1. Pretreatment review to overcome microalgae cell wall resistance

Microalgae present a resilient cell wall mostly composed of organic compounds with slow biodegradability, thereby hampering microalgae degradation. Organic matter is retained in the cytoplasm and is not available for anaerobic microorganisms (González-Fernández et al., 2011). The cell wall structure depends on the microalgae species. Some species such as *Chlamydomonas* or *Euglena* present a glycoprotein fragile cell wall (Cronmiller et al., 2019, Rasul et al., 2017) and others such as *Dunaliella* or *Isochrysis* are naked (D'Hondt et al., 2017), making anaerobic degradation easy. However, generally, eukaryotic microalgae such as *Chlorella, Scenedesmus or Nannochloropsis* present a polysaccharide-based cell wall, mainly composed of cellulose and hemicellulose layers and recalcitrant compounds as sporopollenin and polyterpene that hinder their degradation (Carrere et al., 2016; D'Hondt et al., 2017). Indeed, Mussgnug et al. (2010) investigated six different microalgae species for AD, finding that higher methane yield was associated to microalgae species that either had no cell wall or had a protein-based cell wall, while lower rates were associated with species as *Chlorella* or *Scenedesmus*, that had a polysaccharide-based cell wall. Then, to overcome these drawbacks, several pretreatment strategies have been applied to microalgae in different studies (Carrere et al., 2016; Klassen et al., 2016; Magdalena et al., 2018; Passos et al., 2014a; Passos and Ferrer, 2014) to enhance methane potential and AD rate, improving digester performances. Each pretreatment efficiency relies on microalgal feedstock characteristics such as the cell wall structure and the biochemical composition of the cells. There are different kinds of pretreatments, including: thermal, mechanical, chemical and biological. Pretreatments can be applied independently as single techniques, or they can be combined (two or more methods) to enhance biogas production. A review of recently used pretreatments for microalgae degradation improvement is shown in the next sections and a summary can be found in Table 1.4 and Table 1.5.

Table 1.4. Conditions and results for different thermal and mechanical pretreatments applied to microalgae biomass.

Pretreatment	Conditions	Microalgae	Reactor	Methane yield (untreated microalgae) ($mLCH_4 \cdot gVS^{-1}$)	Methane yield (treated microalgae) ($mLCH_4 \cdot gVS^{-1}$)	Methane yield improve (%)	Reference
Thermal	75 ºC; 10 h	*Chlorella* and *Stigeoclonium*	Batch	90	146	62	Solé-Bundó et al., 2018
	75 ºC; 10 h	*Chlorella* and *Stigeoclonium*	CSTR, 30 d HRT	140	240	71	Solé-Bundó et al., 2018
	70, 90 ºC; 0.5 h	*Chlorella*	Batch	155	215, 228	39, 47	Wang et al., 2017
	121 ºC; 0.3 h			155	322	108	
	120 ºC; 0.33-0.67 h	*Chlorella*	Batch	139*	180*, 268*	30, 93	Mendez et al., 2013
	120 ºC; 0.67 h	*Chlorella*	CSTR, 15 d HRT	85*	126*	49	Mendez et al., 2015
	55 ºC; 15 h	Microalgae biomas[1]	Batch	111	127	14	Passos et al., 2013a
	75 ºC; 15 h			105	160	53	
	95 ºC; 10 h			105	170	62	
	75 ºC; 10 h	Microalgae biomas[2]	CSTR, 20 d HRT	180	300	67	Passos and Ferrer, 2014
	95 ºC; 10 h			180	310	72	

(Continues in next page)

Pretreatment		Conditions	Microalgae	Reactor	Methane yield (untreated microalgae) ($mLCH_4 \cdot gVS^{-1}$)	Methane yield (treated microalgae) ($mLCH_4 \cdot gVS^{-1}$)	Methane yield improve (%)	Reference
Thermal		95 ºC; 10 h 130 ºC; 0.4 h	Microalgae biomass[3]	Batch	106	180 131	70 24	Passos et al., 2015
		70, 90 ºC; 3 h	*Scenedesmus*	Batch	76*	85-170*	12-124	González-Fernández et al., 2012a
		70, 80 ºC; 25 min	*Scenedesmus*	Batch	82*	89-129*	1-57	González-Fernández et al., 2012b
		90 ºC; 1 h	*Scenedesmus*	CSTR, 15 d HRT	33*	97*	193	González-Fernández et al., 2013
Mechanical	*Bead milling*	1-mm glass beads, 4 h	*Scenedesmus*	Batch	54	97	80	Inglesby et al., 2015
		4-mm glass beads, 1 h	*Spirulina*	Batch	133	166	29	Inglesby et al., 2015
		0.35-mm glass beads, 20 min	*Acutodesmus*	Batch	191	289	51	Gruber-Brunhumer et al., 2015.
		1.25-mm, 0.08 h	Microalgae biomass[4]	Batch	162	170	5	Martín et al., 2018
		2.5-mm, 1 h			162	180	11	

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Pretreatment		Conditions	Microalgae	Reactor	Methane yield (untreated microalgae) ($mLCH_4 \cdot gVS^{-1}$)	Methane yield (treated microalgae) ($mLCH_4 \cdot gVS^{-1}$)	Methane yield improve (%)	Reference
Mechanical	*Ultrasound*	39 $kJ \cdot gTS^{-1}$	*Chlorella* (70%),	Batch	336	356	6	Cho et al., 2013
		117 $kJ \cdot gTS^{-1}$	*Scenedesmus*			368	10	
		234 $kJ \cdot gTS^{-1}$	(30%)			385	15	
		20 $kJ \cdot gTS^{-1}$	*Acutodesmus*	Batch	191	292	53	Gruber-Brunhumer et al., 2015.
		16-67 $kJ \cdot gTS^{-1}$	Microalgae biomass[2]	Batch	148	156-196	6-33	Passos et al., 2014a
		26.7 $kJ \cdot gTS^{-1}$	Microalgae biomass[3]	Batch	106	114	8	Passos et al., 2015
		10 $kJ \cdot gTS^{-1}$	*Chlamydomonas*	Batch	272	310	14	Alzate et al., 2012
		27 $kJ \cdot gTS^{-1}$	*Scenedesmus,*			309	14	
		40 $kJ \cdot gTS^{-1}$	*Nannochloropsis*			309	14	
		57 $kJ \cdot gTS^{-1}$				305	12	
		10 $kJ \cdot gTS^{-1}$	*Acutodesmus,*	Batch	198	209	6	Alzate et al., 2012
		27 $kJ \cdot gTS^{-1}$	*Oocystis,*			214	8	
		40 $kJ \cdot gTS^{-1}$	*Phormidium,*			223	13	
		57 $kJ \cdot gTS^{-1}$	*Nitzschia*			223	13	

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	Pretreatment	Conditions	Microalgae	Reactor	Methane yield (untreated microalgae) ($mLCH_4 \cdot gVS^{-1}$)	Methane yield (treated microalgae) ($mLCH_4 \cdot gVS^{-1}$)	Methane yield improve (%)	Reference
Mechanical	*Ultrasound*	10 $kJ \cdot gTS^{-1}$	*Microspora*	Batch	255	314	23	Alzate et al., 2012
		27 $kJ \cdot gTS^{-1}$				301	18	
		40 $kJ \cdot gTS^{-1}$				301	18	
		57 $kJ \cdot gTS^{-1}$				310	22	
		77 $kJ \cdot gTS^{-1}$	*Scenedesmus*	Batch	82*	93*	13	González-Fernández et al., 2012b
		100 $kJ \cdot gTS^{-1}$				144*	76	
		130 $kJ \cdot gTS^{-1}$				154*	88	
	Microwave	22 $kJ \cdot gTS^{-1}$	*Scenedesmus, Chlorella*	Batch	117	150	28	Passos et al., 2013b
		44 $kJ \cdot gTS^{-1}$				167	43	
		65 $kJ \cdot gTS^{-1}$				210	80	
		70 $kJ \cdot gVS^{-1}$	Microalgae biomass[5]	CSTR, 20 d HRT	170	270	58	Passos et al., 2014b
		34.3 $J \cdot gTS^{-1}$	Microalgae biomass[2]	Batch	106	128	21	Passos et al., 2015

1*: Microalgae biomass grown in high rate algal pond mainly composed of Chlamydomonas and Nitzschia although other species as Chlorella, Scenedesmus, Monoraphidium and Ankistrodesmus were also present.*

2: Microalgae biomass grown in high rate algal pond mainly composed of Stigeoclonium, Monoraphidium, Nitzschia and Amphora.

3: Microalgae biomass grown in high rate algal pond mainly composed of Stigeoclonium, Monoraphidium, Nitzschia and Navicula.

4: Microalgae biomass grown in photobioreactor: Tetradesmus (55%), Desmodesmus (16%), Aphanothece (11%), Chlorella (5%), Scenedesmus (4%).

5: Microalgae biomass grown in high rate algal pond mainly composed of Stigeoclonium, Monoraphidium, Scenedesmus and Nitzschia.

**Expressed as $mLCH_4 \cdot gCOD_{inf}^{-1}$*

Table 1.5. Conditions and results for different chemical and biological pretreatments applied to microalgae biomass.

	Pretreatment	Conditions	Microalgae	Reactor	Methane yield (untreated microalgae) ($mLCH_4 \cdot gVS^{-1}$)	Methane yield (treated microalgae) ($mLCH_4 \cdot gVS^{-1}$)	Methane yield improve (%)	Reference
Chemical	*Alkali*	5N NaOH at: pH 9 pH 11 pH 13	*Chlorella* (70%), *Scenedesmus* (30%)	Batch	336	 363 327 213	 8 -3 -58	Cho et al., 2013
	Thermo-alkali	2% NaOH + 50 ºC, 48 h	*Chlorella*	Batch	137*	157*	17	Mahdy et al., 2014c
	Thermo-alkali	5% NaOH + 50 ºC, 24 h	*Scenedesmus*	Batch	141*	170*	20	Mahdy et al., 2014c
	Alkali *Thermo-alkali*	4M NaOH +120 ºC, 20 min	*Chlorella*	Batch	139*	120* 238*	-16 71	Mendez et al., 2013
	Acid *Thermo-acid*	4M H_2SO_4 +120 ºC, 20 min				113* 222*	-23 60	
	Thermo-alkali	0.5N NaOH + 121 ºC, 0.5 h	*Chlorella* *Nannochloropsis*	Batch	340 355	481 432	41 22	Bohutskyi et al., 2014

(Continues in next page)

Pretreatment	Conditions	Microalgae	Reactor	Methane yield (untreated microalgae)	Methane yield (treated microalgae)	Methane yield improve	Reference
				($mLCH_4 \cdot gVS^{-1}$)		(%)	
Biological	Cellulase	*Oocystis* and diatoms	Batch	189	203	8	Passos et al., 2016
	Enzyme mix[1]				217	15	
	Alcalase + 75 ºC, 0.5 h	*Chlorella*	Batch	191*	287*	50	Mahdy et al., 2014a
	Viscozyme + 75ºC, 0.5 h				270*	41	
	Alcalase and viscozyme + 75ºC, 0.5 h				218*	14	
					220*	15	
					270*	41	
					299*	57	
	Alcalase + 75 ºC, 0.5 h	*Chlamydomonas*	Batch	263*	289*	10	Mahdy et al., 2014a
	Viscozyme + 75ºC, 0.5 h				280*	6	
	Alcalase and viscozyme + 75ºC, 0.5 h				255*	-3	
					280*	6	
					289*	10	
					311*	18	
	Viscozyme + 50 ºC, pH 6.5, 5 h	*Scenedesmus*	Batch	206	358	73	Greses, 2017.

1: Enzyme mix (cellulase, glucohydrolase and xylanase).

**Expressed as $mLCH_4 \cdot gCOD_{inf}^{-1}$*

1.4.1.1. Thermal pretreatment

Thermal pretreatments involve biomass heat up in order to solubilise the biomass in a wide range of temperatures (50-270 ºC) and reaction times (from minutes to hours). Thermal pretreatment can be divided into thermal (50-100 ºC) and hydrothermal (>100 ºC) temperature regimes. Passos et al. (2013a) pretreated microalgae biomass at different temperatures, observing that the higher the temperature applied (from 55 to 95 ºC), the higher the methane yield improvement. This is in accordance with González-Fernández et al. (2012a and 2012b), who observed no significant difference when pretreating *Scenedesmus* at 70 ºC but a significant improvement when pretreating microalgae at 80 or 95 ºC (the higher the temperature, the higher the improvement). However, Solé-Bundó et al. (2018) heated microalgae at a constant temperature of 75 ºC for 10 hours, observing that pretreated microalgae methane yield was increased by 62% while Wang et al. (2017) found a lower methane yield improvement pretreating the microalgae biomass at the same temperature, but for a shorter period of time. Wang et al. (2017) also applied a hydrothermal pretreatment (121 ºC), observing a 108% increment in biogas production that is a higher percentage than the observed with thermal pretreatment (47%). On the contrary, Passos et al. (2015) reported 71% improvement when applying a thermal treatment (95 ºC) but 24% improvement when applying a hydrothermal treatment (131 ºC). Then, results found in literature are a bit contradictory, regarding optimum temperature for improving microalgae degradation. Results showed above correspond to batch experiments (Table 1.4) but thermal pretreatment has also been studied in continuous reactors. Solé-Bundó et al. (2018) corroborated their results obtained with batch reactors, reporting a 71% improvement in methane yield when pretreating *Chlorella* and *Stigeoclonium* during 10 hours at 75 ºC in a CSTR. Passos and Ferrer (2014) observed a similar increase (67%) pretreating a microalgae biomass consortium at similar conditions in a CSTR and a slightly higher increase (72%) raising temperature to 95 ºC. González-Fernández et al. (2013) also observed a threefold increase in methane rate treating *Scenedesmus* at 90 ºC for one hour. Nonetheless, Mendez et al. (2015) reported a methane yield enhancement of 50% compared to raw *Chlorella* biomass when treating microalgae at 120 ºC during 40 min in a CSTR but these results were considerably lower than the ones obtained in batch reactors (Mendez et al., 2013). Results from all these studies suggest that, although thermal pretreatment normally gets positive results in terms of methane yield, best temperature and exposure time depends on the

feeding mode or microalgae species digested and, very often, the improvement in methane yield is not enough to offset the energy required for the pretreatment, resulting in a negative energy balance (Cho et al., 2013a; Passos et al., 2013a). Moreover, these methods present other drawbacks such as the formation of recalcitrant compounds that could potentially decrease the reactor's performance, especially when applying hydrothermal temperature (Magdalena et al., 2018; Mendez et al., 2014).

1.4.1.2. Mechanical pretreatment

Mechanical pretreatment consists of disrupting microalgal cells through a physical force. Milling, ultrasounds and microwave pretreatments are the three main mechanical methods that have been used to improve AD of microalgae (Table 1.4).

Bead milling pretreatment has only been studied in batch tests (Table 1.4). It is a complex process and its efficacy relies on duration, bead diameter, bead density, bead filling and agitator speed. Inglesby et al. (2015) reported 80 and 29% enhancement in methane yield when pretreating *Scenedesmus* and *Spirulina*, respectively, stirring 1 L of concentrated algal slurry with glass beads. This improvement was also reported by Gruber-Brunhumer et al. (2015) but they reached an increase of 51% (289 $mLCH_4 \cdot gVS^{-1}$) using milling (100 g of biomass mixed with 40 g of glass beads for 20 min, cooling to 20 °C) for treating *Acutodesmus* with respect to the untreated biomass (191 $mLCH_4 \cdot gVS^{-1}$). However, Martín et al. (2018) observed that, although methane production rate was increased (90% of total methane production was achieved on Day 4), methane yield achieved showed no significant differences applying bead milling pretreatment (Table 1.4).

Ultrasound pretreatment has also been studied in batch reactors. Results have shown how this technique promotes microalgae cell wall disruption and organic matter solubilisation, but the efficiency is highly dependent on the applied specific energy and the type of substrate used and its concentration (Table 1.4). Alzate et al. (2012) applied four energy levels (10 - 57 $kJ \cdot gTS^{-1}$) to pretreat three microalgal mixtures and obtained significant increases in soluble COD in all three. The higher the ultrasound energy applied, the higher the degree of solubilisation. However, the increase in COD solubilisation did not result in a higher methane yield (Table 1.4). The authors reported a maximum of 22% in methane yield improvement when digesting *Microspora* and a maximum of 13% improvement when digesting a mixture of *Chlamydomonas*, *Scenedesmus* and *Nannochloropsis*. This is in accordance with Passos et al. (2015), who found 8% increase

in methane yield when applying 26.7 kJ·gTS^{-1} to microalgae biomass. Nevertheless, Passos et al. (2014a) found that the higher the energy applied, the higher the methane yield, achieving 33% improvement when treating microalgae biomass with 67 kJ·gTS^{-1}. González-Fernández et al. (2012b) found no improvement in methane yield applying energy level of 35.5 and 47.2 kJ·gTS^{-1}, but increments of 13%, 76% and 88% in methane yield were reported for energy levels of 77, 100 and 130 kJ·gTS^{-1}, respectively. Then, results from the above-mentioned studies show that there is considerable variation between reported values, but, overall, it can be concluded that methane yield increase did not exceed 20% with applied specific energy below 75 kJ·gTS^{-1} (Alzate et al., 2012, Gonzalez-Fernandez et al., 2012b) but it was increased by 80-90% when applied specific energy was above 100 kJ·gTS^{-1} (Gonzalez-Fernandez et al., 2012a). Moreover, it has to be highlighted that ultrasound pretreatment requires a high energy input, being a limitation when compared to thermal, chemical or biological methods.

Similar to ultrasonication results, experimental data on microwave pretreatment determined that pretreatment had positive effect on biomass solubilisation and, methane production increased with the applied specific energy. Microwave irradiation enhanced microalgae solubilisation and methane yield under all pretreatment conditions assayed shown in Table 1.4, working with both batch and semi-continuous reactors. Indeed, Passos et al. (2013b) demonstrated that microwave irradiation equivalent to an energy input of 65.4 kJ·gTS^{-1} resulted in a greater increase in methane yield than pretreatment using ultrasound with a similar energy contribution of 67 kJ·gTS^{-1} (Passos et al., 2014b).

1.4.1.3. Chemical pretreatment

Chemical pretreatments for microalgae digestion have been less studied compared to pretreatments described above (thermal and mechanical treatments). Acid and alkali reagents have been commonly used to solubilise polymers (hemicellulose and lignine) favouring the enzymatic attack (Carrere et al., 2016). Chemical treatments are often combined with thermal treatments and so far, all studies have been carried out in batch tests (Table 1.5). For instance, Bohutskyi et al. (2014) found no significant improvement in methane yield when treating *Chlorella* and *Nannochloropsis* with NaOH concentrations ranging from 0 to 20 g·L^{-1}, but, when combining this pretreatment with the thermal one (121 ºC, 10 bar) during 30 min, both *Chlorella* and *Nannochloropsis* microalgae biomass showed a higher solubilisation and methane yield. This is in

accordance with Cho et al. (2013), who only found 8% improvement (from 336 to 363 $mLCH_4 \cdot gVS^{-1}$) when treating a mixture of *Chlorella* and *Scenedesmus* with a solution 5N NaOH at pH 9. However, these authors reported a decrease in methane yield when treating the microalgae mixture with the same solution at pH 11 or 13, reporting 58% decrease at pH 13, suggesting that alkali pretreatment at this pH could have damaged the inoculum activity since the initial pH of the mixture was 10.2. Mahdy et al. (2014c) also observed a methane yield increase when treating *Chlorella* and *Scenedesmus* biomass combining NaOH and a thermal treatment (50 ºC). They studied different NaOH concentrations (0.5, 2 and 5% *w*/*w*) and different time exposure (24 and 40 h). The best conditions for *Chlorella* (2% w/w NaOH at 50 ºC for 48 h) enhanced methane yield by 17%. Likewise, *Scenedesmus* biomass pretreated with 5% NaOH at 50 ºC for 24 h led to 20% methane yield enhancement. Mendez et al. (2013) also observed that applying acid or alkali treatment did not improve methane yield (Table 1.5). Nevertheless, combining it with thermal treatment (120 ºC, 20 min), resulted in a methane yield increase from 139 to 222 $mLCH_4 \cdot gCOD^{-1}$ for thermo-acid treatment and from 139 to 238 $mLCH_4 \cdot gCOD^{-1}$ for thermo-alkali treatment.

Since optimum pH for AD is around 7, one of the main limitation of applying chemical treatments is the need to readjust pH after pretreatment, which leads to a higher cost. Moreover, some of these chemicals need to be removed prior to AD process and need to be combined with thermal pretreatments to obtain significant methane production and solubilisation improvements.

1.4.1.4. Biological pretreatment

One alternative to high energy-demanding pretreatments (thermal, mechanical and thermo-chemical treatments) is the use of biological reactions, which show high selectivity and absence of external inhibitory compounds. The enhancement of digestion and methane rate via enzymatic hydrolysis of microalgal cell wall and other polymers has been proven to be effective (Greses, 2017; Klassen et al., 2016). Hydrolytic enzyme pretreatments with enzymes mixtures (proteases, cellulases, xylanases and β-glucanases) have been used for algal solubilisation (Klassen et al., 2016). Their efficiency depends on the cell wall composition, enzyme dose, substrate specificity, temperature, exposure time and pH.

Greses (2017) reported 73% improvement in methane yield when treating *Scenedesmus* with a carbohydrolase enzyme (Viscozyme). Mahdy et al. (2014) found that the addition of enzymes carbohydrolase and protease resulted in high carbohydrates and protein solubilisation in *Chlorella* and *Chlamydomonas* biomass. However, carbohydrolase addition did not result in any methane improvement for *Chlamydomonas* microalgae and 14% improvement for *Chlorella* biomass. On the other hand, the addition of protease resulted in 50% increase in methane yield treating *Chlorella* and a slight increase (18%) for *Chlamydomonas* biomass. The best scenario for both microalgae biomass was observed when combining the addition of protease and carbohydrolase with thermal pretreatment (Table 1.5). Passos et al. (2016) treated microalgae biomass (*Oocystis* and diatoms) with cellulase and an enzyme mix (cellulase, glucohydrolase and xylanase), reporting an increase in biomass solubilisation: 110% after enzymatic pretreatment with cellulase and 126% after enzyme mix. Regarding methane yield, 8% improvement was associated with cellulase treatment and 15% with enzyme mix treatment. Then, as seen in studies reported above, the mixture of enzymes improved the performance with respect to a single enzyme. As all these biological pretreatments have been evaluated at batch scale, they should be further evaluated in continuous reactors to know the efficiency of the treatment and the feasibility for industrial application.

1.4.2. Review on C/N ratio effects on microalgae digestion

The substrate C/N ratio is a key factor for the stability of an AD process. A high C/N ratio could result in N deficiency for biomass synthesis while a high C/N ratio could cause inhibition problems due to an excess of N. Even different C/N ratios have been recommended in literature for AD feedstock, a range between 20/1 - 30/1 and an optimum of 25/1 has been generally reported for a balanced nutrient supply (Angelidaki et al., 2003; Li et al., 2011; Lin et al., 2019; Yen and Brune, 2007). Many studies reported that C/N ratio for microalgae biomass ranges from 4/1 to 15/1 (typically below 10) (Herrmann et al., 2016; Tran, 2017; Kwietniewska and Tys, 2014; Yen and Brune, 2007), which are unfavourable ratios for AD process. The low C/N ratio of microalgae due to their high N content results in high total ammonia nitrogen (TAN) released during AD. Ammonia is one of the most common AD inhibitors (Kougias and Angelidaki, 2018). It is well documented that the toxic ammonia effects are attributed to the free NH_3 and not to ammonium ion (NH_4^+) but it is difficult to establish an inhibition threshold since ammonia

toxicity relies on several factors such as pH, temperature, TAN concentration or inoculum source. For instance, the higher the temperature or pH, the higher the free ammonia nitrogen (FAN) released. Inhibition caused by FAN accumulation in the digester could lead also to VFA accumulation, which will in turn decrease the pH of the system. This pH decrease will partially mitigate the FAN toxicity, since its concentration diminishes with pH. However, this mechanism will keep the system in an "inhibited steady state" condition that affect the reactor performance and diminish methane yield (Kougias and Angelidaki, 2018). Indeed, VFA and FAN accumulation could result in complete failure of the process (Kinnunen et al., 2014; Sung and Liu, 2003).

One alternative to avoid ammonia accumulation is to adjust the low microalgae C/N ratio. One method for raising C/N ratio reported in literature is cultivating microalgae under nitrogen starvation (Klassen et al., 2015). This strategy resulted in lower protein content (Klassen et al., 2015; Klassen et al., 2017; Markou et al., 2013). Klassen et al. (2015) reported an increase in C/N ratios up to 24-26 for three tested microalgal strains (*Chlamydomonas reinhardtii*, *Parachlorella kessleri* and *Scenedesmus obliquus)* when limiting the nitrogen content available for microalgae in batch cultures. Klassen et al. (2017) observed a highly accessible biomass for anaerobic microorganisms with two times lower protein content when cultivating microalgae in a semi-continuous reactor. However, these experiments were based on synthetic culture media in which a desired nutrients concentration is applied, but in a real WRRF in which nutrients from wastewater need to be recovered, this technique is not feasible. Other alternative to increase C/N ratio is co-digesting microalgae biomass with carbon-rich substrates.

1.5. ANAEROBIC CO-DIGESTION OF MICROALGAE WITH CARBON-RICH SUBSTRATES

Co-digestion, also known as co-fermentation, is a process in which different wastes are digested simultaneously to overcome the drawbacks of mono-digestion and to obtain a higher AD yield. Co-digesting microalgae with other substrates have several advantages: not only the C/N ratio can be improved, but also the system's buffer capacity, the dilution of inhibitory compounds, the nutrient balance, the digestion performance and biogas yield (Saratale et al., 2018). For this reason, research on this field has increased notably in the last two decades (Saratale et al., 2018). Numerous studies have demonstrated that co-digesting microalgae with waste substrates increases biogas yield because of the positive

synergism formed in the AD medium. Table 1.6 shows some examples of studies carried out in microalgae anaerobic co-digestion (ACoD).

Table 1.6. Microalgae co-digestion with different carbon-rich substrates.

Co-substrates	**Operation**	**Proportions**	**Methane yield** $mLCH_4 \cdot gVS^{-1}$	**Reference**
Scenedesmus and *Chlorella* + waste paper (WP)	CSTR, 35 ºC	100% M	143	Yen and Brune, 2007
		75% M, 25% WP	242	
		50% M, 50% WP	292	
		25% M, 75% WP	79	
		100% WP	112	
Microcystis + corn straw (CS)	CSTR, 35 ºC	100% M	160	Zhong et al., 2013
		65% M, 35% CS	234	
Arthrospira + beet silage (BS), barley straw (bs) or seaweed (S)	BMP, 37 ºC	100% M	357	Herrmann et al., 2016
		45% M, 55% BS	361	
		85% M, 15% bs	348	
		15% M, 85% S	311	
Chlorella and *Scenedesmus* + food waste (FW)	BMP, 35 ºC	100% M	107	Zhen et al., 2016
		80% M, 20% FW	285	
		60% M, 40% FW	381	
		50% M, 50% FW	525	
		40% M, 60% FW	549	
		20% M, 80% FW	639	
		100% FW	575	

M: Microalgae

Yen and Brune (2007) observed how methane production was increased when co-digesting microalgae (*Scenedesmus* and *Chlorella*) with waste paper (WP) in proportions of 75-25 or 50-50, respectively. Digesters fed with 50% microalgae and 50% WP showed a methane yield increased from 143 to 292 $mLCH_4 \cdot gVS^{-1}$, compared to microalgae mono-digestion. Also, digesters TAN levels decreased with the increased feedstock C/N ratio. However, when WP fraction in feedstock increased up to 75%, the AD process showed instability and methane yield decreased. The increase in C/N ratio to 36/1 at 75% WP and 25% microalgae seemed to be excessive for a good AD performance. Then, the authors suggested an optimal C/N ratio of 20-25/1 for AD process. Zhong et al. (2013) observed 46% improvement in methane yield, with less accumulation of FAN and VFA when co-digesting microalgae (*Microcystis*) with corn straw compared to microalgae mono-

digestion. In accordance, Zhen et al. (2016) observed a higher methane yield compared to microalgae (*Chlorella* and *Scenedesmus*) mono-digestion, co-digesting microalgae biomass with food waste (FW). Co-digestion of 80% FW and 20% microalgae achieved 5-fold methane production increase comparing to digestion of microalgae alone, exceeding also the methane production of FW alone and thus indicating a synergetic effect between both substrates. However, Herrman et al. (2016) studied the ACoD of *Arthrospira* microalgae with different substrates (beet silage, barley straw and seaweed) and no synergistic effect on methane production was found. Then, several substrates such as FW or WP, among others, have been reported to improve microalgae digestibility (Table 1.6). However, the transport cost of the co-substrate from the generation point to the AD plant is one of the most important criteria when choosing the adequate co-substrate. Therefore, co-digesting microalgae with other kind of substrate such as sewage sludge, that is being produced in the same WRRF, would save transporting costs. Nonetheless, choosing the co-substrate and its proportion in feedstock should also be based on the methane yield optimisation, the positive effect of synergisms and the dilution of harmful compounds.

1.5.1. Anaerobic co-digestion of microalgae with sewage sludge

There are several research papers on co-digestion of microalgae with various types of sludge (Table 1.7). For instance, various authors have studied the ACoD of microalgae with waste activated sludge (WAS). Wang et al. (2013) observed that methane yield obtained co-digesting WAS and *Chlorella* was higher than that obtained digesting microalgae alone, but, this methane yield did not exceed the yield from WAS digestion alone. However, Olsson et al. (2014) observed that methane yield obtained co-digesting microalgae biomass (*Chlorella*) and WAS in proportions of 37% and 63%, respectively, was higher than the value observed digesting both substrates alone (Table 1.7). This is in accordance with Beltrán et al. (2016), who observed that the higher the WAS fraction added to microalgae (*Chlorella* and *Scenedesmus*), the higher the methane yield, and reported that methane production observed co-digesting both substrates was higher than that observed digesting each substrate alone (Table 1.7). Mahdy et al. (2015b) also studied the ACoD of *Chlorella* biomass with WAS. Raw microalgae biodegradability was higher than WAS biodegradability and, when both substrates were co-digested, methane yield remained low. Indeed, these authors characterised both substrates (microalgae and WAS)

chemically and observed quite similarities between them. Then, results found in literature regarding WAS and microalgae co-digestion are a bit contradictory (Table 1.7).

Table 1.7. Microalgae co-digestion with different kinds of sewage sludge.

Co-substrates	Operation	Proportions	Methane yield ($mLCH_4 \cdot gVS^{-1}$)	Reference
Chlorella + waste activated sludge (WAS)	BMP, 37 ºC	100% M	124	Wang et al., 2013
		41% M, 59% WAS	296	
		11% M, 89% WAS	272	
		4% M, 96% WAS	298	
		100% WAS	302	
Chlorella +WAS	BMP, 37 ºC	100% M	368	Olsson et al., 2014
		37% M, 63% WAS	408	
		100% WAS	332	
	55 ºC	100% M	319	
		37% M, 63% WAS	323	
		100% WAS	366	
Chlorella + WAS	BMP, 35 ºC	100% M	109*	Mahdy et al., 2015b
		75% M, 25% WAS	107*	
		25% M, 75% WAS	92*	
		100% WAS	81*	
Chlorella + primary sludge (PS)		75% M, 25% PS	171*	
		25% M, 75% PS	252*	
		100% PS	265*	
Chlorella and *Scenedesmus* + WAS	BMP, 35 ºC	100% M	318	Beltrán et al., 2016
		75% M, 25% WAS	354	
		50% M, 50% WAS	380	
		25% M, 75% WAS	442	
		100% WAS	362	
Chlorella + PS	BMP, 35 ºC	100% M	90	Solé-Bundó et al., 2018
		75% M, 25% PS	133	
		50% M, 50% PS	216	
		25% M, 75% PS	291	
		100% PS	378	
Chlorella + PS	CSTR, 37 ºC	100% M	200	Solé-Bundó et al., 2019
		25% M, 75% PS	333	

M: Microalgae

**Methane yield is expressed as $mLCH_4 \cdot gCOD_{inf}^{-1}$*

Mahdy et al. (2015b) also studied the ACoD of microalgae with primary sludge and the authors observed that primary sludge showed a high biodegradability and, when co-digesting it with microalgae, methane yield was higher than that observed digesting microalgae alone. Then, Mahdy et al. (2015b) reported that WAS did not improve microalgae digestibility but primary sludge did. Solé-Bundó et al., (2018 and 2019) who studied ACoD of microalgae (*Chlorella*) with primary sludge in both batch and semi-continuous experiments also observed a higher methane production when microalgae were co-digested with primary sludge, however, microalgae biodegradability was not improved, then, further research regarding microalgae and primary sludge co-digestion is needed.

1.6. MICROBIAL ANALYSIS IN ANAEROBIC DIGESTION SAMPLES

The analysis of microbial communities in bioengineering systems can provide valuable information for a better understanding of biological processes taking place in the system and then, for optimising operating parameters that affect process performance. Microbial analysis offers a link between biodiversity, operating conditions and digester efficiency. AD was known as "black-box" due to the insufficient knowledge about microorganisms involved in this process and their metabolisms. Nevertheless, several authors have studied the microbial core of AD in the last decades (Rivière et al., 2009, Maus et al., 2016), and more recently, microbial communities involved in microalgae AD have also been studied (Greses, 2017, Klassen et al., 2017, Zamorano et al., 2019a). These authors reported the importance of saccharolytics hydrolysers and fermenters, as well as proteolytic bacteria for microalgae cell-wall disruption. However, even microorganisms involved in microalgae co-digestion with different substrates have been studied, only a few studies have analysed the microorganisms involved in a co-digestion system with sewage sludge (Lee et al., 2017; Zamorano et al., 2019b) and only at mesophilic temperature.

A fundamental step to know microbial community involved in AD processes is the phylogenetic and taxonomic classification of DNA sequences. A wide pool of molecular tools can be used for characterising microbial core. High-throughput sequencing (HTS) technologies (massive sequencing techniques) have been extensively used in the last decades for their capacity of sequencing multiple DNA molecules in parallel. There are several HTS techniques, the one developed by Illumina (USA) being one of the most used in the last decade. This technique is based on clonal amplification by bridge polymerase

chain reaction (PCR) and sequencing-by-synthesis (specifically, sequencing by reversible termination). A basic workflow for an Illumina HTS includes four basic steps: library preparation, cluster generation, sequencing and data analysis. Data obtained after Illumina sequencing need a computational downstream processing. With this aim, different bioinformatics pipelines have been studied and used during the last decades. Among them, quantitative insights of microbial ecology (QIIME), developed by Caporaso et al. (2010) and *Mothur* (Schloss et al., 2009) are the most reported in literature. Both methods allow a filtering of the raw genes sequences obtained from Illumina or other sequencing platforms, offering a clean sequence of the target gene, free of chimeras, homopolymers and replicated sequences. For microbial analysis in AD processes, the small subunit of rRNA sequences (16S rRNA) is the common targeted DNA fragment. Once gene 16S rRNA sequences have been obtained and microorganisms involved in AD process have been identified, biostatistics techniques such as multivariate analysis can be applied to link microbial ecology with bioengineering parameters that are key for digesters operation such as SRT, HRT or organic loading rate (OLR). 16S rRNA gene amplicon sequencing has been extensively used to investigate microbial community structure and dynamics in anaerobic digesters, offering valuable information for understanding processes occurring in AD and improving reactor's operation.

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Thesis plan

2. THESIS PLAN

The doctoral thesis is structured in 9 chapters:

Chapter I: In this chapter, a general introduction and a theoretical background on the thesis' topic is given. A research on the current state of the art into the microalgae anaerobic digestion and co-digestion can be found in this chapter.

Chapter II: This chapter includes the explanation of the thesis structure for a better understanding. This chapter also offers the aim and motivation of the doctoral thesis, including the partial objectives necessaries to reach the global aim.

Chapter III: In this chapter, a description of materials and methods used for the thesis development can be found. Experimental design and analytical methods are described, as well as the calculations associated with the experiments.

Chapter IV, V, VI, VII: This thesis is presented in a paper format composed of four published chapters, so these four chapters offer the experimental results of the PhD thesis and are structured in the typical paper format, including: introduction, material and methods, results and discussion, conclusions and references.

Chapter VIII: This chapter offers an overall discussion of the PhD thesis, compiling results from all experiments carried out during the thesis.

Chapter IX: This thesis ends with a chapter including general conclusions of the thesis work and future perspectives on the field of microalgae co-digestion.

2.1. AIMS AND OBJECTIVES

The global aim of this thesis is the long-term evaluation of the ACoD process of raw microalgae and primary sludge in an AnMBR for obtaining valuable resources from wastewater. Furthermore, this work aims to evaluate biological and filtration process performance, joint to the microbial community involved in the process. Different parameters for the co-digester operation were tested at laboratory-scale and best operating conditions were chosen for scaling-up the process to pilot-scale.

The specific objectives of each chapter are summarized below:

Chapter IV:

- To evaluate microalgae and primary sludge ACoD at lab-scale working at a high SRT of 100 d and mesophilic conditions (35 ºC).
- To analyse the stability of biological process and microbial community in two connected mesophilic reactors, acting the second reactor as a biomass reservoir.
- To characterise microbial community involved in ACoD: differences between inoculum and first and second mesophilic reactors.

Chapter V:

- To evaluate microalgae and primary sludge ACoD at lab-scale working at thermophilic conditions (55 ºC).
- To evaluate free ammonia toxicity in a thermophilic CSTR and in a thermophilic AnMBR.
- To assess membrane implementation to a thermophilic CSTR.
- To determine incidence on microbial community after implementing a membrane in the system.
- To determine incidence of increasing OLR on biological process and microbial community.

Chapter VI:

- To evaluate microalgae and primary sludge ACoD at lab-scale working at a 70 d SRT and mesophilic conditions (35 ºC) in order to study the incidence of SRT over ACoD.
- To study the scale-up from laboratory to pilot-scale of microalgae and primary sludge ACoD at mesophilic conditions.
- To carry out an energy assessment of ACoD working at mesophilic and thermophilic temperature. To compare the results obtained with the ones obtained from Chapter V.
- To carry out an economic assessment of ACoD working at an SRT of 100 d and an SRT of 70 d. To compare the results obtained with the ones obtained from Chapter IV.
- To assess filtration process at laboratory and pilot-scale. To study different parameters over filtration performance.

Chapter VII:

- To evaluate biogas production and nutrients recovery from ACoD process at pilot-scale.
- To study composting process at lab-scale. Comparison between composting of a sludge coming from the ACoD pilot plant and a sludge coming from a conventional digester.
- To assess the effect of different conditions over composting process.
- To evaluate nitrogen recovery from ACoD pilot plant's effluent.
- To evaluate phosphorus recovery from ACoD pilot plant's effluent.

Material and Methods

3. MATERIAL AND METHODS

3.1. SUBSTRATES FOR ANAEROBIC CO-DIGESTION TRIALS

Harvested microalgae and primary sludge were used as substrates for ACoD trials at laboratory and pilot-scale. Microalgae were collected from a MPBR pilot plant operated by Calagua Research Group in the Cuenca del Carraixet WWTP (Alboraya, Valencia, Spain). This MPBR plant was fed with the effluent of an AnMBR pilot plant, also operated in the Carraixet WWTP. The AnMBR pilot plant, which is extensively described in Giménez et al. (2011), treated wastewater coming from the Carraixet WWTP gravity-based primary clarifier. This plant consisted of an anaerobic reactor (AnR) with a volume of 1300 L connected to two membrane tanks (MTs). The AnMBR treatment offers a solids-free permeate but it does not remove nutrients. Then, the MPBR plant uses this nutrient-rich effluent for microalgae cultivation. The MPBR plant consisted in two flat-plane methacrylate photobioreactors (PBRs) connected to a MT (PBR1-PBR2) and two other PBRs (PBR3-PBR4) that were not connected to the MT. PBR1 and PBR2 had a volume of 230 L and PBR3 and PBR4 had a volume of 550 L. The dimensions of the four MPBR were equal in height and weight but their depth was different (PBR1 and PBR2 were 0.10 m deep while PBR3 and PBR4 were 0.25 m deep). An extensive description of the MPBR pilot plant and its operation can be found in González-Camejo (2019). Microalgae culture was mainly dominated by *Chlorella* and *Scenedesmus* (>99% of the total eukaryotic cells). To achieve the desired COD to be fed to the co-digester, harvested microalgae were concentrated in an external cross-flow filtration tubular module containing ultrafiltration membrane fibres (HF 5.0-43-PM500, ROMICON® Koch Membrane Systems, USA), with a pore size of 500 kDa molecular weight cut-off and a filtration area of 2.1 m^2. Primary sludge, used as co-substrate in this study, came from the Carraixet WWTP gravity thickener and was sieved through a 0.5 mm sieve to discard large particles. After that, the sludge was diluted in order to adjust the COD concentration to the desired value with the purpose of keeping constant the OLR. Once both substrates were prepared, they were characterised and stored at 4 ºC during a maximum of 3 weeks. The complete scheme of all units described can be seen in Figure 3.1.

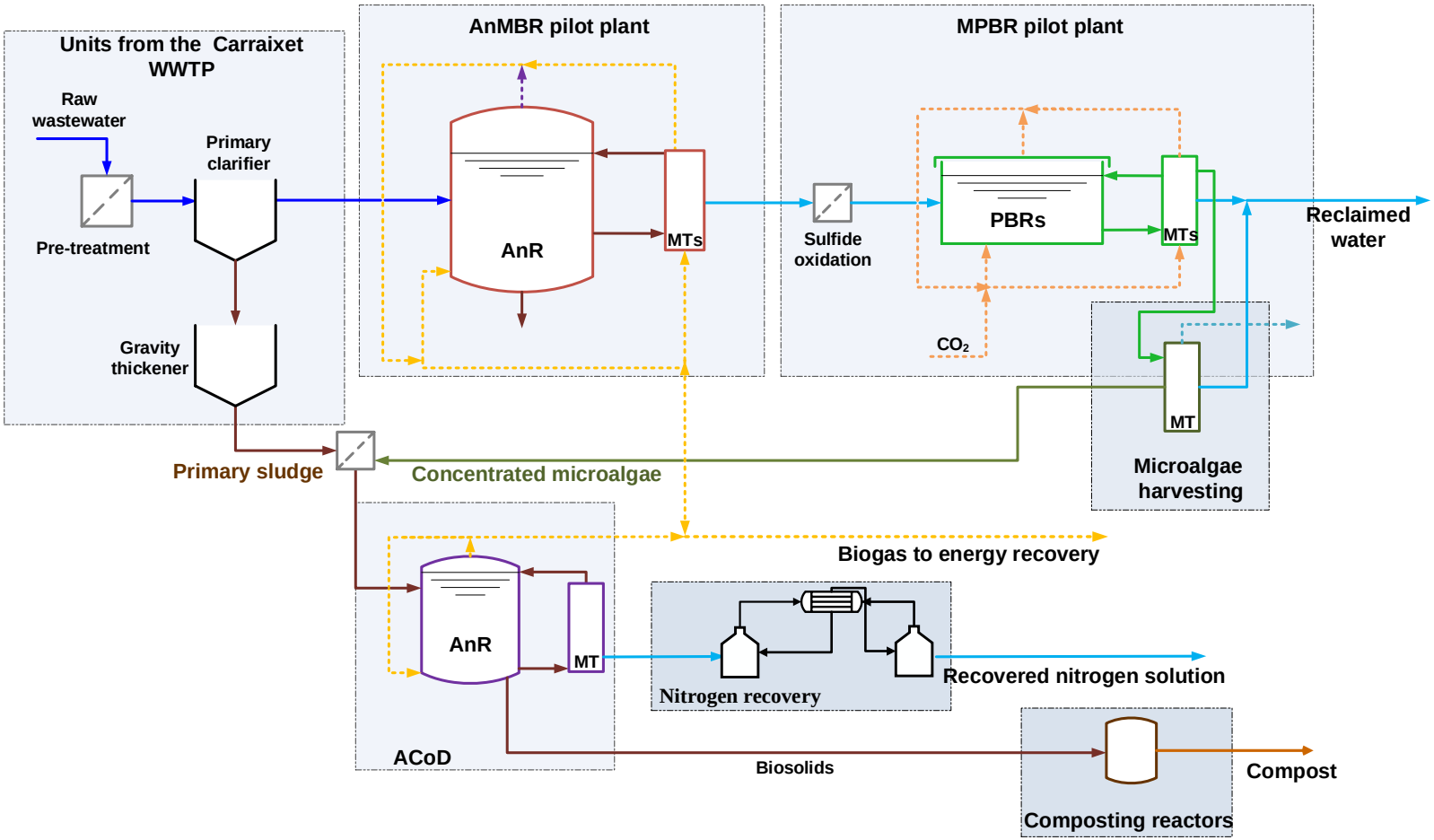

Figure 3.1. Complete layout of all units being part of a scheme for resource recovery from urban wastewater through microalgae and primary sludge anaerobic co-digestion process. AnR: anaerobic reactor; AnMBR: anaerobic membrane bioreactor; ACoD: anaerobic co-digestion; MTs: membrane tanks; PBRs: photobioreactors

3.2. EXPERIMENTAL SET-UP AND OPERATING CONDITIONS FOR ANAEROBIC CO-DIGESTION TRIALS

ACoD of microalgae and primary sludge was studied at laboratory and pilot-scale in an AnMBR. Layout of the experimental set-up was similar at both scales.

3.2.1. Lab-scale operation

ACoD experiments at lab-scale were carried out under different operating conditions of temperature, SRT and OLR. Mesophilic and thermophilic temperatures were evaluated. Experimental set-up for mesophilic or thermophilic experiments was similar, with differences in the biomass mixing method or the type of membrane used for filtration process. Pseudo-steady state was considered to be reached when some parameters such as biogas production or total solids (TS) and nutrient concentrations showed almost no variation for at least around one SRT and process was then considered stable and reproducible.

3.2.1.1. Mesophilic operation

The experimental set-up for the continuous ACoD trials at mesophilic temperature consisted of a cylindrical methacrylate CSTR with a total volume of 14 L and a working volume of 9 L (Figure 3.2). The reactor was connected to an external hollow-fibre ultrafiltration membrane (UHF) module (PURON Koch membrane systems, 0.03 μm pore size) with a surface area of 0.42 m^2, a total volume of 1 L and a working volume of 0.9 L. Sludge was kept homogenized by continuous recirculation through the UHF, where the effluent was obtained by vacuum filtration. Two biogas blowers (ZR P4 24 V dc, EAD, Spain*)* were connected in order to allow mixing of both the reactor and the membrane module and to control biofilm formation on the membrane. One blower recycled a fraction of the biogas produced in the AnMBR reactor from top to bottom, while the other blower recycled another biogas fraction from the top of the reactor to the bottom of the UHF. An MGC-1 milligas-counter (Ritter, Germany) was used to measure daily volumetric biogas production. Gas volumes were normalised to conditions of pressure and temperature of 1 atm and 0 °C. Reactor was kept at 35 °C by a water jacket connected to a thermostatic water bath. The system was sealed to ensure anaerobic conditions and avoid gas leakage and was equipped with pH, redox and temperature electrodes. The headspace reactor and transmembrane pressure (TMP) were also

monitored and all values were recorded by a custom-written data-logging script in visual basic.

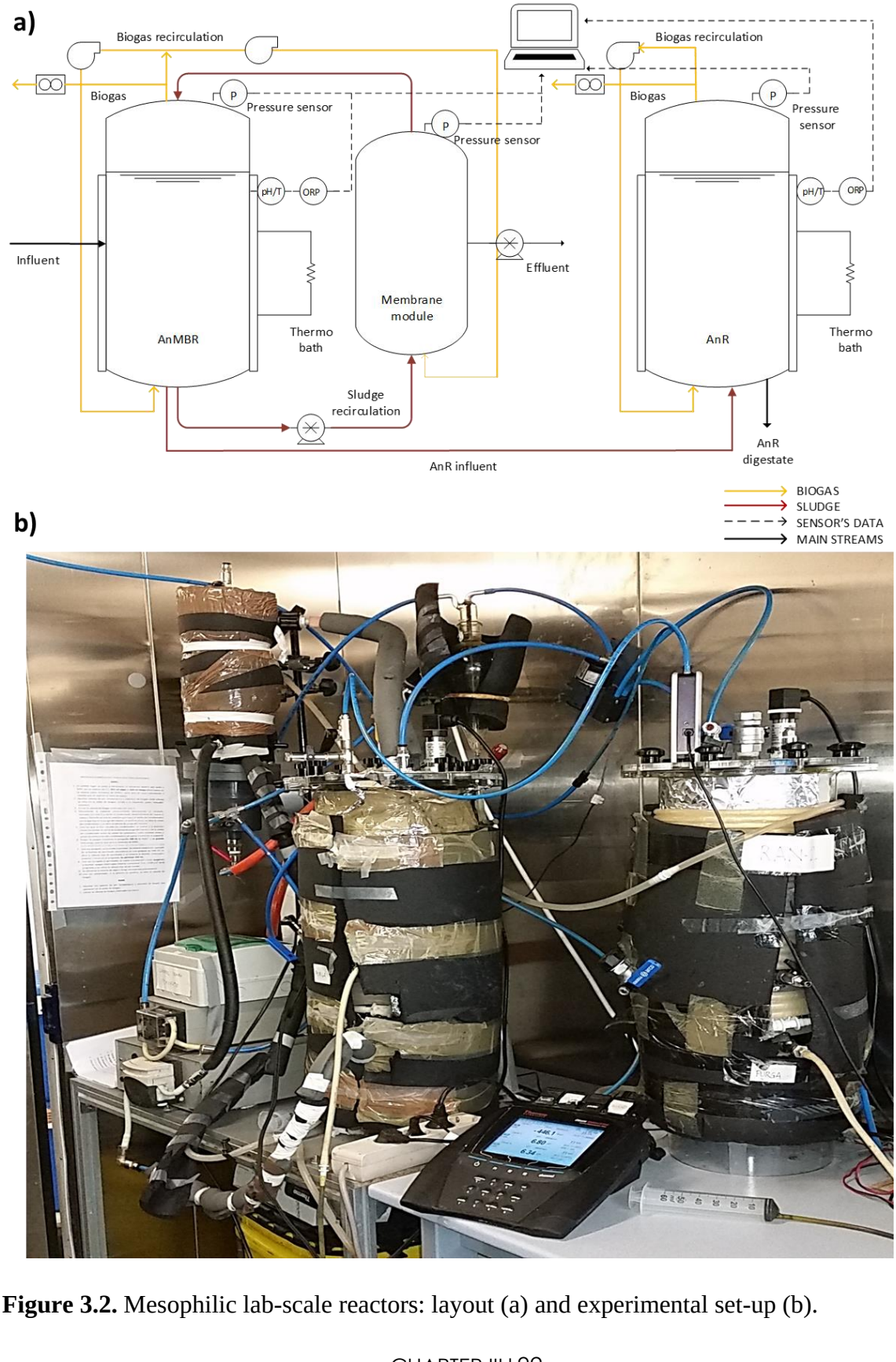

Figure 3.2. Mesophilic lab-scale reactors: layout (a) and experimental set-up (b).

The system was run during 830 days, divided into two periods (Periods PI and PII) keeping all operating conditions equals, except for the SRT (Table 3.1). SRT was set in 100 d in Period PI, which lasted 440 days and then was set at 70 d in the second experiment, which lasted 390 days (Table 3.1). The reactor was fed manually, using plastic syringes, once a day. The substrate composition was constant during both periods: 62% primary sludge and 38% microalgae based on VS. This ratio was chosen according to the results of a co-digestion pilot plant simulation (using DESSAS® software) to reproduce the mass proportion produced in a WWTP combining an AnMBR plant treating settled wastewater followed by an MPBR plant as a secondary treatment (Figure 3.1). Mesophilic AnMBR was inoculated with 7 L of sludge from a lab-scale mesophilic reactor digesting microalgae biomass.

Table 3.1. Operating conditions for mesophilic reactors at lab-scale.

	Mesophilic AnMBR		**Mesophilic AnR**
Period	PI	PII	PI
SRT (d)	100	70	100
HRT (d)	30	30	100
OLR ($gCOD \cdot L^{-1} \cdot d^{-1}$)	0.52	0.5	0.15
Temperature (ºC)	35	35	35
Membrane	Yes	Yes	No
Microalgae biomass	*Chlorella* and *Scenedesmus*	*Chlorella*	*Chlorella* and *Scenedesmus*
Days of operation	440	390	440

SRT: solids retention time; HRT: hydraulic retention time; OLR: organic loading rate; AnMBR: anaerobic membrane bioreactor, AnR: anaerobic reactor.

With the aim of having a biomass reservoir, a second mesophilic reactor, connected to the AnMBR was also evaluated during the first period (PI). This second reactor had the same characteristics as mesophilic AnMBR but lacked a membrane (Figure 3.2). It was also connected to a blower to allow the correct mixing of the reactor through biogas recirculation. The purge stream from the mesophilic AnMBR was fed to the reactor 2 (AnR). Then, mesophilic AnR was inoculated (filled) with the daily purge from the AnMBR. As the biomass was not recirculated in the AnR, SRT and HRT were set at 100 days. This AnR thus offers a biomass reservoir and a two-stage system providing a total

SRT of 200 d (100 d in the AnMBR plus 100 d in the AnR) in the system during Period PI. A scheme of both reactors can be seen in Figure 3.2.

3.2.1.2. Thermophilic operation

A lab-scale CSTR, similar to the mesophilic reactor, was used for AD under thermophilic conditions (Figure 3.3). The biomass was mechanically stirred and temperature was kept at 55 ºC by means of a water jacket. The reactor had a total and working volume of 13 L and 9 L, respectively.

The system was run for 700 days divided into four periods (PI, PII, PIII and PIV). The reactor was inoculated with 9 L of sludge from a lab-scale mesophilic anaerobic digester treating microalgae and primary sludge. The reactor was fed once a day with the same mixture than mesophilic reactor (62% primary sludge and 38% microalgae based on VS). During the start-up (first 45 days of operation), the reactor was operated as a CSTR, at an OLR of 0.22 $gCOD \cdot L^{-1} \cdot d^{-1}$; hence, SRT was equal to HRT (70 d). A ceramic ultrafiltration membrane module (0.1 μm pore size Likuid Nanotek, Spain) was installed on Day 46 and allowed an SRT of 70 days while keeping HRT at 30 days during all periods investigated. The layout of the AnMBR is shown in Figure 3.3. The progressive increase of OLR was studied from Periods PI to PIV (Table 3.2).

Table 3.2. Operating conditions for thermophilic reactor at lab-scale.

		Thermophilic AnMBR			
Period	**Start-up**	**PI**	**PII**	**PIII**	**PIV**
SRT (d)	70	70	70	70	70
HRT (d)	70	30	30	30	30
OLR ($gCOD \cdot L^{-1} \cdot d^{-1}$)	0.22	0.17	0.30	0.40	0.50
Temperature (ºC)	59	55	55	55	55
Membrane	No	Yes	Yes	Yes	Yes
Microalgae biomass	*Chlorella*	*Chlorella*	*Chlorella*	*Chlorella*	*Chlorella*
Days of operation	0-45	46-253	254-423	424-471	472-700

SRT: solids retention time, HRT: hydraulic retention time; OLR: organic loading rate; AnMBR: anaerobic membrane bioreactor

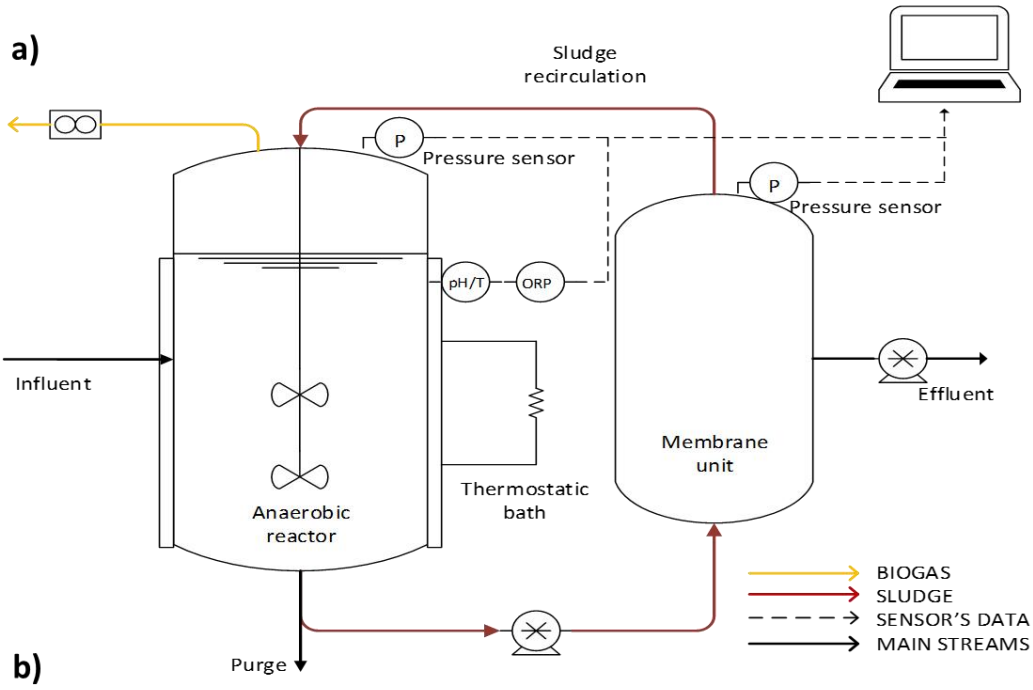

Figure 3.3. Thermophilic lab-scale reactor: layout (a) and experimental set-up (b).

3.2.2. Pilot-scale operation

For pilot-scale ACoD trials, the experimental set-up was very similar to the one used at laboratory-scale (Figure 3.4). The co-digestion pilot plant consisted of a reactor with a total volume of 1010 L and a working volume of 910 L, coupled to a 1 L MT fitted with a 0.44-m^2 UHF unit (0.03 μm pore size, PURON® KMS, USA). The different co-substrates (microalgae and primary sludge) were mixed in a 125 L equalisation tank (ET) before being fed to the system. A 70 L purge tank (PT), a 70 L foam trap (FT) and a 42 L clean-in-place (CIP) tank, where the permeate was accumulated, were also included as main elements of the plant. To control the digester's temperature (35 ºC), three external resistances were installed in the reactor's wall. Total biogas produced was measured by a biogas meter (BK-G4M, Elster, USA) and gas volumes produced were normalised to pressure and temperature conditions of 1 atm and 0 ºC, respectively. A fraction of the generated biogas was recycled from the top of the reactor to the bottom, employing a blower (HX10 05 P1 230V 50/60Hz, EAD, Spain) to assure the correct mixing of the sludge and to facilitate the stripping of the gases from the liquid phase. Another fraction of the biogas produced was recycled through another blower from the top of the reactor to the bottom of the UHF, minimising the biofilm formation. Sludge was continuously recycled through the MT where the effluent was obtained by vacuum filtration, after passing through a degasification vessel (DV).

Different sensors were used for process monitoring: pH, temperature and ORP electrodes, four level transmitter (one for each tank, except for the foam tramp), a liquid-flow transmitter in the MT permeate, a gas-flow transmitter in the biogas inflow to the MT, two liquid pressure transmitters to determine TMP in the MT, a biogas pressure transmitter in the co-digester headspace and a rotameter in the biogas inflow to the co-digester.

The pilot plant was run for 360 days and operating conditions were kept equal to the ones used at laboratory scale during period PII: OLR of 0.5 $gCOD \cdot L^{-1} \cdot d^{-1}$, SRT of 70 d and HRT of 30 d. The ACoD pilot plant was inoculated with anaerobic sludge coming from the Carraixet full-scale mesophilic digester. The system was fed automatically every day. To control the SRT and HRT in the system, a fraction of the sludge and permeate were extracted from the co-digester throughout the day.

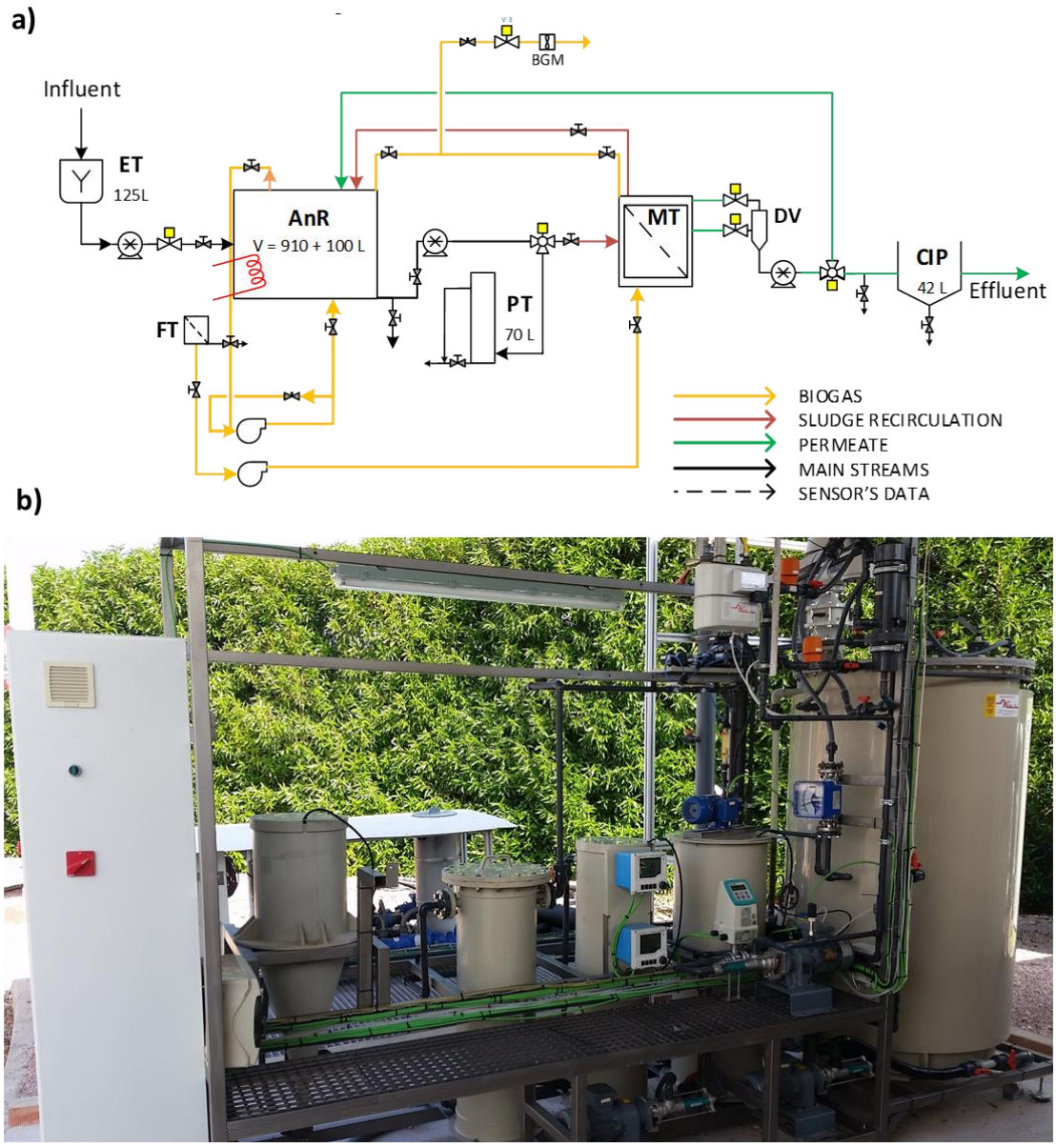

Figure 3.4. Anaerobic co-digestion pilot plant: layout (a) and experimental set-up (b). ET: equalisation tank; AnR: anaerobic reactor; PT: purge tank; MT: membrane tank; DV: degasification vessel; CIP: Clean in Place tank; BGM: biogas meter.

3.3. MEMBRANE OPERATION

The membrane was operated both at laboratory and pilot-scale. In laboratory reactors, both mesophilic and thermophilic, filtration was carried out discontinuously: a basic filtration-backwash-relaxation cycle was performed once per day in order to obtain enough permeate to maintain the desired HRT, and relaxation state was performed during the rest of the day. In the pilot plant, filtration was carried out continuously. However, to

establish the influence of several factors over filtration performance, several experiments were carried out in the mesophilic co-digester both at laboratory and pilot-scale.

3.3.1. Lab-scale operation

Filtration experiments at lab-scale were carried out in the mesophilic AnMBR during the last 30 days of period PII. Membrane was operated at a specific gas demand (SGD) of 0.15 $N \cdot m^3 \cdot m^{-2} \cdot h^{-1}$ and a permeate flux normalised at 20 ºC (J_{20}) of 5.8 $L \cdot m^{-2} \cdot h^{-1}$ (LMH). The filtration and backwash times were 90 and 40 s, respectively and relaxation period was removed for these laboratory trials. Then, a backwash was conducted every filtration cycle.

Filtration TMP and permeability were assessed periodically at three different fluxes (3.5, 7.0 and 10.5 LMH) twice a week. The evaluation consisted in setting the J_{20} during three filtration cycles and determining the maximum TMP and permeability under these conditions.

3.3.2. Pilot-scale operation

Pilot plant filtration was carried out at an average J_{20} of 3.5 LMH and a filtration time of 180 s during the first 260 days of operation. The membrane operation was automated to enable the study of various relaxation and backwash frequencies and durations. A basic cycle of filtration-relaxation (F-R) was established and taken as a basis to schedule the frequency of the remaining stages. Two additional stages were also considered to remove the accumulation of biogas at the top of the fibres (degasification) and inside the DV (ventilation). During the last 100 days of operation, the pilot-scale filtration was performed under four different conditions (Experiments I to IV, Table 3.3). A membrane physical cleaning consisting of pressure washing was realised after each experiment. While filtration times were different in the experiments carried out, relaxation and backwash times were 30 s and 45 s, respectively in all experiments. A backwash was conducted every F-R cycle in Experiments I and II, but every two cycles in Experiments III and IV.

Table 3.3. Operating conditions for the four filtration experiments carried out at pilot-scale.

	Specific gas demand ($N \cdot m^3 \cdot m^{-2} \cdot h^{-1}$)	**Permeate flux** ($L \cdot m^{-2} \cdot h^{-1}$)	**Recirculation ratio**	**Filtration time** (s)
Experiment I	0.6244 ± 0.0005	5.6 ± 0.3	1.81 ± 0.00	300
Experiment II	0.151 ± 0.002	5.5 ± 0 .7	1.7 ± 0.2	160
Experiment III	0.15 ± 0.02	4.18 ± 0.04	1.92 ± 0.05	160
Experiment IV	0.32 ± 0.01	4.22 ± 0.03	1.81 ± 0.04	160

3.4. EXPERIMENTAL SET-UP FOR NUTRIENTS RECOVERY

3.4.1. Composting process

3.4.1.1. Substrates for composting process

Composting process of ACoD sludge was evaluated at lab-scale. ACoD sludge came from the ACoD mesophilic pilot plant (Section 3.2.2). The ACoD sludge composting process was compared to a conventional sludge (coming from the digester of the full-scale Carraixet WWTP) composting process. Reactors fed with ACoD sludge are named in the present document as R_A and reactors fed with conventional sludge are named as R_C. Both sewage sludges were pretreated in a centrifuge to remove the excess of moisture, achieving values around 80-87% of moisture. Conventional sludge was dried in the Carraixet WWTP industrial centrifuge. ACoD sludge was dried in a lab-scale centrifuge (Eppendorf, Centrifuge 5804), at 4350 rpm for 30 minutes and after that, the obtained pellet was centrifuged for 15 minutes more. In addition, ACoD sludge was left to air-dry for 48 hours before its use.

To ensure optimal conditions for composting, different BAs were characterised, to choose the best one to be added to the above-mentioned sludges (Table 3.4). Due to its high carbon content and its availability in the University of Valencia's garden, pruning remains were chosen as BA in this study. Remains were ground to achieve the correct size to be assimilated by microorganisms involved in the process.

Table 3.4. Five bulking agent characterisation.

Bulking agent	C/N ratio	Nitrogen (%)	Moisture (%)
Pruning remains	49.55	0.90	49.10
Lawn	13.89	3.08	78.95
Olive wood	109.4	0.42	48.25
Cypress tree	58.40	0.81	57.89
Orange tree	29.38	1.50	30.62

The relative proportions of ACoD sludge or conventional sludge and BA were calculated according to two methods: "volumetric proportions" and "theoretical proportions". For volumetric proportions, 2.5 L of BA were added per each litre of sludge (conventional or ACoD sludge). Theoretical proportions were calculated setting the C/N ratio in a value of 25 and the moisture content in a value between 50% and 70%, according to Eq.3.1.

$$\frac{m_{BA}}{m_s} = \frac{(25 \cdot N_S(kg)) - C_S(kg)}{C_{BA}(kg) - (25 \cdot N_{BA}(kg))} \quad \text{(Eq. 3.1)}$$

where m_{BA} and m_s are the proportions of BA and sludge respectively, N_s and C_s are the nitrogen and carbon contents, respectively, in the sludge and N_{BA} and C_{BA} are the nitrogen and carbon contents, respectively, in the BA.

3.4.1.2. Experimental set-up and operating conditions

Composting experiments were carried out in 7 reactors (R_{A1}-R_{A4}; R_{C1}-R_{C3}). These composting reactors were glass Dewar flasks (KGW Isotherm Germany), with a volume of 4 L, especially designed for composting process (Figure 3.5). Dewar flasks were covered by an aluminium coating that offered a high thermal isolation. Covers of Dewar flasks were perforated to allow gas evacuation. A plastic mesh was incorporated at the bottom of the reactors and was covered by a gravel layer to allow a correct separation between leachate and composted material. A temperature probe was included in each reactor for daily measuring temperature.

Reactors were operated around a maximum of 44 days, in pairs, to evaluate the effect of different parameters over composting process. Three parameters were assessed: i) aeration, ii) addition of inoculum and iii) mixture between BA and sludge. Table 3.5 shows the main operating conditions of the seven reactors.

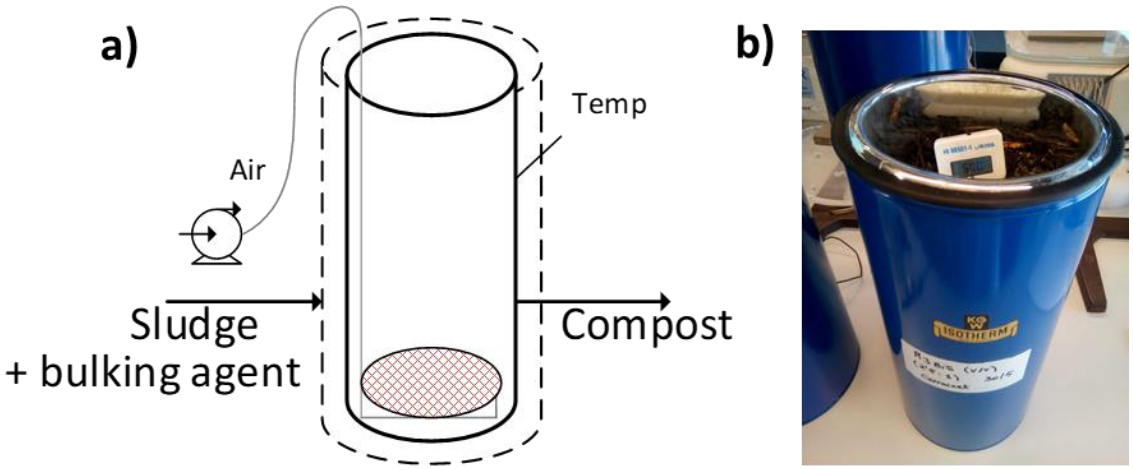

Figure 3.5. Composting reactor: layout (a) and experimental set-up (b).

The effect of aeration was studied running the pairs of reactors R_{A1} and R_{A3} and R_{C1} and R_{C3}, which were run with the same operating conditions but changing aeration mode: R_{A1} and R_{C1} received forced aeration while R_{A3} and R_{C3} were turned by hand (Table 3.5). The effect of adding inoculum to the reactor was assessed operating the pair of reactors R_{A2} and R_{A4}, which had the same operating conditions but reactor R_{A2} had inoculum while reactor R_{A4} had absence of inoculum (Table 3.5). Finally, the effect of mixing different proportions of sludge with BA was evaluated operating pairs of reactors R_{A2} and R_{A3} and R_{C2} and R_{C3}, which were run with the same operating conditions but with different proportions of sludge and BA (Table 3.5).

Table 3.5. Operating conditions for the seven reactors studied.

Reactors identification	Days in operation	Sludge	Mixture proportions	Aeration	Inoculum
R_{A1}	36	ACoD	Theoretical	Forced	✓
R_{C1}	36	Conventional	Theoretical	Forced	✓
R_{A2}	44	ACoD	Volumetric	Turned by hand	✓
R_{C2}	44	Conventional	Volumetric	Turned by hand	✓
R_{A3}	27	ACoD	Theoretical	Turned by hand	✓
R_{C3}	27	Conventional	Theoretical	Turned by hand	✓
R_{A4}	31	ACoD	Volumetric	Turned by hand	-

ACoD: anaerobic co-digestion; A: ACoD sludge; C: conventional sludge

Reactors R_{A1} and R_{C1} received forced aeration from the bottom of the reactors to the top by an air compressor with a flow rate of 2 $L \cdot min^{-1}$. The rest of the reactors were manually turned once a day. In all reactors, except R_{A4} (Table 3.5), 400 mL of inoculum from the maturation stage of a compost pile from Vintena´s composting plant (Carcaixent, Valencia) were added to the mixture inside the reactor with the purpose of accelerating the speed reaction of the microorganisms involved in the process.

3.4.2. Nitrogen recovery

For nitrogen recovery from AnMBR permeate, a hydrophobic polypropylene hollow-fibre membrane contactor (HFMC) (X50 2.5x8 Liqui-Cel®, USA) was used at laboratory-scale. The HFMC had a surface of 1.4 m^2 and was connected to two closed tanks of 1.2 L where the permeate and the acid solution were stored. Each tank was equipped with pH and temperature electronic sensors (SP10T, Consort®, Belgium) connected to a multiparametric analyser (Consort® C832, Belgium). The acid stream (0.05 M H_2SO_4) circulated in the lumen side at a flow rate of 0.4 $L \cdot min^{-1}$ while the permeate was fed in the shell side at a flow rate of 0.6 $L \cdot min^{-1}$ and a pH of 10.

3.5. SAMPLING AND ANALYTICAL METHODS

3.5.1. Anaerobic co-digestion experiments

Reactors and substrates samples for ACoD trials were regularly analysed. TS, VS, Alkalinity (Alk) and VFA were determined three times a week in triplicate. TS and VS concentrations were determined according to method 2540-B and 2540-E, respectively, of Standard Methods (APHA, 2012). VFA and Alk determination was carried out using the 5-points acid-base titration method proposed by Moosbrugger et al. (1992). Other parameters such as Total COD (T-COD), Soluble COD (S-COD), Total Suspended Solids (TSS), and Volatile Suspended Solids (VSS) were measured in triplicate once a week. TSS and VSS concentrations were determined according to methods 2540-D and 2540-E of Standard Methods (APHA, 2012). T-COD and S-COD were performed according to Standard Methods (APHA, 2012). Phosphate (PO_4-P), Sulphate (SO_4-S), and Ammonium (NH_4-N) were measured also in duplicate once a week using an automatic analyser (Smartchem 200, Westco Scientific Instruments, USA), according to methods 4500-P-F, 4500-SO_4^{2-}-F and 4500-NH_3-G from Standard Methods (APHA, 2012). Total Phosphorus (TP), and Total Nitrogen (TN) were measured in duplicate once a week. TN concentration

was measured by colorimetric analysis using the nitrogen total cell test kit (Merckoquant 1.14537.001, Merck, Germany). TP concentration was measured after an acid digestion of the sample at 150 ºC during two hours (4500-P-B), followed by orthophosphate determination according to Standard Methods, 4500-P-F (APHA *et al.*, 2012).

The co-digesters headspace methane content was measured three times per week by a gas chromatograph equipped with a flame ionization detector (GC-FID, Agilent Technologies, USA), and a TRACER column (Teknokroma, Spain): 15 m × 0.53 mm ×1 μm. The temperatures of the detector and the column were kept at 250 and 40 °C, respectively. A flow rate of 5 $mL \cdot min^{-1}$ was used for the carrier gas (helium). The concentration of the sample gas was contrasted with a standard pure gas (99.9995% methane).

The C, N, H, and S composition of microalgae and primary sludge samples was analysed by energy-dispersive X-ray spectroscopy (EDS) by means of an XL-30 ESEM (Philips, Netherlands). Substrate samples were attached to the Scanning Electron Microscopy (SEM) stub using silver lacquer. The SEM stub with the sample was then introduced into the XL-30 and the pressure was reduced to 10^{-5} bar, after which the sample surface was visualised and an area was selected for microanalysis. The spot-size value was modified until a dead time of around 30% was achieved.

To assess the filtration process in the lab-scale mesophilic AnMBR, the viscosity and SMPs content in mixed liquor of the co-digester were evaluated twice a week. The amount of SMPs was attributed only to the protein and carbohydrate concentrations. Viscosity was determined by a Cannon-Fenske viscometer (Series 200, Merck, Germany). Carbohydrate concentration was determined using the Dubois method (Dubois et al., 1956). Proteins concentration was determined using a commercial Total Protein Kit, Micro Lowry, Peterson's Modification (Sigma-Aldrich, USA), based on the Lowry-Peterson method (Peterson, 1977).

Microscopic analysis for total eukaryotic cells determination in microalgae samples was carried out by epifluorescence microscopy on a Leica DM2500. Total eukaryotic cells concentration was measured in duplicate in each microalgae sample. 50 μL of sample were filtered with 0.2 μm membranes (Millipore GTTP, USA). The filters were first washed with distilled water to eliminate the retained salt and then dehydrated with

successive ethanol washes. Cell counts were performed using the 100x-oil immersion lens. A minimum of 300 cells were counted and at least 100 cells of the most abundant genera were counted with an error of less than 20% (Pachés et al., 2012).

3.5.2. Composting experiments

To assess the composting process performance, C/N ratio, TN and porosity of both ACoD sludge and conventional sludge mixtures with BA were determined in initial composting samples. In addition, presence of pathogens (*E. coli* and *Salmonella* spp.) in both sludges (before being mixed with BA) was measured. Organic matter and moisture content, pH and electric conductivity were weekly monitored in composting reactor's samples. For final composted product, C/N ratio and pathogens of each mixture were also analysed.

Moisture and organic matter content, pH and electric conductivity were measured according to Standard Methods (APHA 2012) with the corresponding dilutions for adapting the method procedure to compost samples. For instance, for electric conductivity and pH determination, the sample was previously diluted in a ratio of 1:10. After 30 min of agitation and 20 min of centrifugation (11000 rpm), the supernatant was analysed.

C/N ratio was determined by measuring the elemental components of the composting samples using the Elemental Analyser EA 1110 CHNS (CE Instruments Ltd, Wigan, United Kingdom). A previous pretreatment of the sample, which consisted in drying the sample at 65 ºC in an oven and applying a milling process, was carried out to transform the heterogeneous material in a homogenous powder. Porosity was determined by the weight difference between the original sample and the sample saturated with water. TN in composting samples was determined according to APHA (2012) with a previous homogenisation of the sample in a sonicator (S250D, Branson) and a later dilution in a ratio 1:1000.

E. coli presence was quantitatively determined by the standard method for enumeration of *E. coli* β-glucuronidase positive, following the UNE-EN ISO 9308-1:2014. *Salmonella* spp. was measured following the UNE-EN ISO 19250 standard method.

3.6. MICROBIAL POPULATION ANALYSIS

Microbial population was analysed in laboratory reactors: mesophilic AnMBR and AnR during PI and thermophilic AnMBR during all periods at laboratory scale.

3.6.1. Sampling and nucleic material extraction

3.6.1.1. Mesophilic reactors

Inoculum and digestate samples were pelleted after centrifuging 1 mL of sludge at 4000 x g and stored at -20 ℃ until DNA extraction procedures. Besides the inoculum, three samples were collected from the AnMBR and AnR after 286, 323 and 400 days of operation. All these samples correspond to the pseudo-steady period and were therefore considered biological replicates. A negative control with sterilized and nuclease-free deionized water was also included at the beginning of the DNA extraction procedure. Following the protocol described by Zamorano-López et al. (2019) the nucleic acid material was extracted from all the samples and used for 16S rRNA amplicon sequencing.

3.6.1.2. Thermophilic reactor

A total of 17 samples (Table 3.6) were extracted from the thermophilic reactor. Samples #1 to #2 were extracted during the start-up and #3 to #4 during PI to monitor the microbial community evolution after start-up. Samples #5 to #17 (excluding #11, #12, #13 and #14) were extracted when pseudo-steady state was achieved (i.e. 3 samples in each of the four study periods) and can thus be considered biological replicates. Samples #11, #12, #13 were extracted during PIII before achieving pseudo-steady state. Sample #14 was extracted during an operational problem in period PIV to monitor its effect on the microbial community. Pellets of 1 mL digestate samples were stored in 2 mL cryotubes at −20 °C. The E.Z.N.A DNA Extraction Kit for Soil (Omega-Biotek, USA) was used to extract nucleic acid material from 1 g of biomass, in accordance with the manufacturer's protocol. A Nanodrop 2000 spectrophotometer (Thermo Scientific, USA) was used to determine DNA concentration and purity through the absorbance measured at wavelengths of 260, 230 and 280 nm. To avoid RNA, humic acids or other compounds contamination, only sequences with an A260/280 ratio over 1.8 and an A260/230 ratio between 2.0 and 2.2 were sequenced.

Table 3.6. Sample collection for 16 rRNA gene sequencing in the thermophilic reactor.

Period	Sample Number	Day of operation	Comments
Start-up	#1 #2	17 and 36	Before membrane installation
PI	#3 #4	50 and 64	After membrane installation
	#5 #6 #7	232, 238 and 247	Pseudo-steady state
PII	#8 #9 #10	263, 276 and 290	Pseudo-steady state
PIII	#11 #12 #13	466, 470 and 472	Transient state
PIV	#14	552	After an operational problem
	#15 #16 #17	650, 662 and 670	Pseudo-steady state

3.6.2. Sequencing

16S rRNA gene analysis of bacteria and archaea microorganisms of all reactors 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 805R 5'- GACTACHVGGGTATCTAATCC -3′) (Klindworth et al., 2013). The sequencing run was carried out in a 2×300 base pairs paired-end run using v3 chemistry in an Illumina MiSeq Sequencer from the sequencing service "*Fundación para el Fomento de la Investigación Sanitaria y Biomédica de la Comunitat Valenciana*" (FISABIO, Valencia, Spain). The resulting raw sequences were deposited on the NCBI platform in the Sequence Reads Archive (SRA) depository under Bioproject PRJNA434206.

3.6.3. Illumina data processing

Since different methods are available for processing the sequences retrieved from Illumina amplicon sequencing, two different methods were used for mesophilic or thermophilic data processing.

Downstream amplicon sequencing analysis for mesophilic reactors' samples was performed according to a previous study (Zamorano-López et al. 2019) in QIIME pipelines and R-Studio software plus *vegan* and *mixOmics* packages. The resulting

filtered sequences were clustered into Operational Taxonomic Units defined at 3% dissimilarity ($OTU_{0.97}$).

The resulting raw sequences from thermophilic samples sequencing were downstream processed to remove chimeras following the *MiSeq_SOP* pipeline (website accession data was 21st June 2019) using open-source *Mothur* software (v.1.42). The taxonomy was assigned according to the v132 release of SILVA database.

3.6.4. Statistical analysis

Biodiversity in mesophilics and thermophilic reactors was calculated through the evenness index indicators: Shannon-Wiener and Simpson Evenness Estimator, which account alpha diversity considering species richness. A principal co-ordinate analysis (PCoA) based on the Bray-Curtis distances matrix was performed to explore the beta diversity of the different samples collected from the AnMBRs and AnR. Subsampling was applied to the minimum level of sequences obtained for both alpha and beta diversity.

3.7. CALCULATIONS

3.7.1. Anaerobic co-digestion process performance

The ACoD process efficiency was evaluated in terms of biodegradability percentage, biomethane potential percentage, methane yield, COD removal, VS removal, and ammonia concentration using equations 3.2 to 3.7.

$$Biodegradability = \frac{CH_4 \sim COD + H_2S \sim COD}{COD_{inf}} \cdot 100\ (\%) \quad \text{(Eq. 3.2)}$$

$$Biomethane\ potential = \frac{CH_4 \sim COD}{COD_{inf}} \cdot 100\ (\%) \quad \text{(Eq. 3.3)}$$

$$Y^{CH_4} = \frac{CH_4 \sim V}{COD_{inf}} \left(\frac{L\ CH_4}{g\ COD}\right) \quad \text{(Eq. 3.4)}$$

$$CODremoval = \frac{COD_{inf} - COD_{eff}}{COD_{inf}} \cdot 100\ (\%) \quad \text{(Eq. 3.5)}$$

$$VS\ removal = \frac{VS_{inf} - VS_{eff}}{VS_{inf}} \cdot 100\ (\%) \quad \text{(Eq. 3.6)}$$

$$[NH_3] = \frac{TAN}{1+\frac{10^{-pH}}{10^{-(0.09018+\frac{2728.92}{T})}}} \quad \left(\frac{mg}{L}\right) \qquad \text{(Eq. 3.7)}$$

where COD_{inf} (g COD·d^{-1}) is the influent COD; CH_4~COD is the COD of the produced methane (biogas methane and methane dissolved in the effluent (calculated according to Eq. 3.8) (g COD·d^{-1}); H_2S~COD is the COD consumed by SRB for sulphate reduction (g COD·d^{-1}), COD_{eff} is the effluent COD (g COD·d^{-1}), VS_{inf} is the influent VS concentration (g VS·d^{-1}), VS_{eff} is the effluent VS concentration (g VS·d^{-1}), TAN is the total ammonia nitrogen in the reactor (mg·L^{-1}), T is the temperature expressed in Kelvin and CH_4~V is the volume of methane produced (biogas methane plus dissolved methane in the effluent) at 1 atm and 0 ºC (L).

Dissolved methane in the effluent was calculated following the Henry's law (Eq. 3.8), according to the method previously reported by Giménez (2014).

$$P \cdot y_{CH_4} = H_{CH_4}(T) \cdot x_{CH_4} \qquad \text{(Eq. 3.8)}$$

where P is the pressure (atm), y_{CH4} is the molar concentration of the gas, H_{CH4} is the Henry's law constant for methane and x_{CH4} is the molar concentration of the gas in liquid. H_{CH4} depends on temperature (T, in Kelvin) and it is calculated according to Eq. 3.9 (Tchobanoglous et al., 2003)

$$H_{CH_4}(T) = 10^{\left(\frac{-675.76}{T}+6.88\right)} \qquad \text{(Eq. 3.9)}$$

3.7.2. Filtration process performance

The filtration process efficiency was evaluated through the 20 ºC-normalised membrane permeability (K_{20}) according to Eq. 3.10. Since permeate viscosity affects the filtration performance, a temperature correction factor (f_T) was included in the model (Eq. 3.11), which can be also used to determine the 20 ºC-normalised permeate flux (J_{20}) (Eq. 3.12).

$$K_{20} = \frac{J_T \cdot f_T}{TMP} \ (LMH \cdot bar^{-1}) \qquad \text{(Eq. 3.10)}$$

$$f_T = e^{-0.0239\,(T-20)} \qquad \text{(Eq. 3.11)}$$

$$J_{20} = J \cdot f_T \ (LMH) \qquad \text{(Eq. 3.12)}$$

where J is the permeate flux, T is temperature, in Kelvin and TMP is the transmembrane pressure.

3.7.3. Nutrients recovery

Efficiency in nutrients recovery after ACoD process was evaluated using Eq. 3.13 and 3.14.

$$\%\ P\ recovery\ efficiency = \frac{P_{eff}}{P_{inf} + P_{rel}} \cdot 100 \quad \text{(Eq. 3.13)}$$

$$\%\ N\ recovery\ efficiency = \frac{TAN_{eff,0} - TAN_{eff,end}}{TAN_{eff,0}} \cdot 100 \quad \text{(Eq. 3.14)}$$

where P_{eff} (mgP-$PO_4 \cdot L^{-1}$) is the phosphate concentration in the effluent, $P_{inf,}$ (mgP-$PO_4 \cdot L^{-1}$) is the phosphate concentration in the influent, P_{rel} (mgP-$PO_4 \cdot L^{-1}$) is the phosphate released into the reactor during AD, $TAN_{eff,0}$ (mgN-$NH_4 \cdot L^{-1}$) is the initial concentration of TAN in the hollow-fibre membrane contactor (HFMC) influent (permeate from ACoD pilot plant) and $TAN_{eff,end,}$ (mgN-$NH_4 \cdot L^{-1}$) is the TAN concentration at the end of the process (HFMC effluent).

3.7.4. Composting process

Respirometric assays were carried out according to Barrena et al. (2005) to monitor biological activity. Barrena et al. (2005) indicated that respirometric assays at the in situ temperature are representative of the metabolic state of microorganisms, so static respirometric assays at process temperature were carried out in those reactors achieving thermophilic temperature to monitor biological activity of the process. Nevertheless, Barrena et al. (2005) also indicated that assays at 37 ºC are more useful to study the stability of the process. Hence, for monitoring the biological activity of the composting material, static respirometric assays at a fixed temperature of 37 ºC were also performed in the same reactors to analyse the stability of the mixture. For the calculation of the respirometric index (RI), the slope of the oxygen concentration (%) versus time curves was calculated for each assay. RI calculation was carried out according to Barrena et al. (2005), following Eq. 3.15.

$$RI = \frac{V \cdot P \cdot 32 \cdot m \cdot 60}{R \cdot T \cdot X \cdot DM \cdot OM} (mgO_2 \cdot g\ organic\ matter^{-1} \cdot h^{-1}) \quad \text{(Eq. 3.15)}$$

where V is the volume of air in flask (mL), P is the atmospheric pressure (atm), m is the slope of change in percent O_2 saturation per minute divided by 100; R is the ideal gas constant ($atm \cdot mol^{-1} \cdot K^{-1}$), T is the temperature (K), X is the wet weight of compost test sample (g), DM is the TS fraction of the compost sample and OM is the organic matter fraction of the compost sample in dry basis ($gOM \cdot gDM^{-1}$).

3.7.5. Energy and economic assessment

An energy and economic balance was performed according to the model proposed by Seco et al. (2018), to select the best operating conditions for scaling-up the process from laboratory to pilot-scale, working at mesophilic conditions. For energy balance, only heating requirements were considered. The heating requirements (Q_{total}) were calculated considering the energy necessary for increasing the influent temperature and the reactor's heat losses (Eq. 3.16).

$$Q_{total} = c_P \cdot q \cdot \rho_{sludge} \cdot \left(T_{reactor} - T_{inflow}\right) + U \cdot S \cdot \left(T_{reactor} - T_{outside}\right) \left(\frac{kcal}{h}\right) \quad \text{(Eq. 3.16)}$$

where c_P ($kcal \cdot kg^{-1} \cdot K^{-1}$) is the specific heat; q ($m^3 \cdot h^{-1}$) is the input flow rate; ρ_{sludge} ($kg \cdot m^{-3}$) is the sludge density; ($T_{reactor}$-T_{inflow}) (K) is the temperature difference between the inflow and the reactor; U ($kcal \cdot h^{-1} \cdot m^{-2} \cdot K^{-1}$) is the overall heat transfer coefficient obtained by Eq. 3.17; S (m^2) is the reactor surface, and ($T_{reactor}$-$T_{outside}$) (K) is the temperature difference between the reactor and the outside.

$$U = \frac{1}{\frac{\delta_{reactor}}{K_{reactor}} + \frac{1}{h_{air}}} \left(\frac{kcal}{h \cdot m^2 \cdot K}\right) \quad \text{(Eq. 3.17)}$$

where $\delta_{reactor}$ (m) is the reactor thickness; $K_{reactor}$ ($kcal \cdot h^{-1} \cdot m^{-1} \cdot K^{-1}$) is the reactor material conductivity, and h_{air} ($kcal \cdot h^{-1} \cdot m^{-1} \cdot K^{-1}$) is the air convective heat transfer coefficient.

Heat recovery was calculated as the heat recovered from methane considering a combined heat and power (CHP) unit (Q_{CHP}; Eq. 3.18). Since the amount of dissolved methane in the effluent was low and no treatment was designed for its recovery, it was not considered in this calculation.

$$Q_{CHP} = \frac{V_{BG} \cdot \left(\%CH_4 \cdot CV_{CH_4}\right) \cdot \%q_{eff\ CHP}}{1000 \cdot 24 \cdot 4.187} \cdot \%q_{exch} \left(\frac{Kcal}{h}\right) \quad \text{(Eq. 3.18)}$$

where V_{BG} ($L \cdot d^{-1}$) is the biogas volume, $\%CH_4$ is the methane richness; CV_{CH4} ($kJ \cdot m^{-3}$) is the methane calorific power; $\%q_{eff}$ CHP is the heat efficiency of the CHP system, and

$\%q_{exch}$ is the heat exchanger efficiency. A q_{eff} CHP of 45% and a q_{exch} of 65% were considered according to Seco et al. (2018).

Total energy demand (Q_{demand}) was calculated according to Eq. 3.19.

$$Q_{demand} = (Q_{TOT} - Q_{CHP}) \cdot 0.0011622 \left(\frac{kWh}{m^3}\right) \quad \text{(Eq. 3.19)}$$

Reactor constructing costs (C_C), equipment costs (including biogas blowers, recirculating pumps, permeate pumps and purge pumps), land cost as well as depreciation costs (D_C) were considered for economic balance calculations, according to Eq. 3.20 and 3.21.

$$C_C = Concrete\ wall\ price \cdot V_R + Concrete\ slab\ price \cdot V_C\ (k€) \quad \text{(Eq. 3.20)}$$

where concrete wall price and concrete slab price are the prices of the materials needed for the reactor construction (k€·m^{-3}), V_R is the annular volume of the reactor (m^3) and V_C is the volume of the reactor cover (m^3).

$$D_C = \frac{C_C + Equipment\ cost + Land\ cost}{t}\ (k€) \quad \text{(Eq. 3.21)}$$

where t is the depreciation period (years). In this work, a depreciation period of 20 years was chosen for calculations.

For both energy and economic balance, some considerations relating to reactor characteristics and construction materials were assumed for different operation scales (Table 3.7). Electricity production from produced biogas (W_{BG}) was also considered (Eq. 3.22).

$$W_{BG} = \frac{V_{BG} \cdot \%\ CH_4 \cdot CV_{CH4} \cdot \eta_{CHP}}{1000 \cdot 24 \cdot 3600}\ (kW) \quad \text{(Eq. 3.22)}$$

where η_{CHP} is the methane electricity conversion efficiency by the CHP system. A η_{CHP} of 0.35 was considered according to Seco et al. (2018).

Table 3.7. Considerations for energy and investment balance at different scales.

	Lab-scale	Pilot-scale	Industrial-scale
Input flow ($m^3 \cdot d^{-1}$)	0.00046	0.03	2014
Reactor thickness (m)	0.05	0.15	0.5
Reactor height (m)	0.4	2.2	13
Reactor diameter (m)	0.19	1	26
$K_{reactor}$ ($kcal \cdot h^{-1} \cdot m^{-1} \cdot K^{-1}$)	0.03	0.03	1.40
Energy (€·kWh^{-1})	-	-	0.11*
Material for construction	PVC	PVC	Concrete
Concrete wall (€·m^{-3})	-	-	350
Concrete slab (€·m^{-3})	-	-	130

$K_{reactor}$: reactor material conductivity; PVC: polyvinyl chloride
**Aura Energía (2020)*

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Co-digestion of harvested microalgae and primary sludge in an anaerobic membrane bioreactor (AnMBR): methane potential and microbial diversity

4. CO-DIGESTION OF HARVESTED MICROALGAE AND PRIMARY SLUDGE IN A MESOPHILIC ANAEROBIC MEMBRANE BIOREACTOR (AnMBR): METHANE POTENTIAL AND MICROBIAL DIVERSITY.

Abstract: Anaerobic co-digestion of primary sludge and raw microalgae (*Scenedesmus* and *Chlorella*) was performed in a lab-scale semi-continuous anaerobic membrane bioreactor to assess the biological performance and identify the microbial community involved in the co-digestion process. The reactor was operated at 35 °C for 440 days, working at a solids retention time of 100 days. The system achieved 73% biodegradability and showed high stability in terms of pH and volatile fatty acids. An enriched microbial community was observed. Of the several phyla, Chloroflexi and Proteobacteria were the most abundant. Cellulose-degraders phyla (Bacteroidetes, Chloroflexi and Thermotogae) were detected. Syntrophic microorganisms played an important role in intermediate degradation, enhancing methane production, mainly carried out by *Methanosaeta*. A nutrient-rich effluent (400 $mgNH_4\text{-}N \cdot L^{-1}$ and 29 $mgPO_4\text{-}P \cdot L^{-1}$) and digestate (860 $mgN \cdot L^{-1}$ and 151 $mgP \cdot L^{-1}$) were obtained. The bio-nutrients released from anaerobic co-digestion could be reused for microalgae cultivation or agricultural applications.

Keywords: co-digestion; AnMBR; microalgae; primary sludge; methane

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4.1. INTRODUCTION

Expanding energy consumption due to industrialization and population growth has encouraged the scientific community to search for alternative renewable and eco-friendly energy sources to substitute fossil fuels. Thanks to its potential for nutrient removal or recovery from wastewater streams, microalgae cultivation has received increasing interest in recent years (Viruela et al., 2016) and has also attracted the attention of the bioenergy field. Microalgae are a valuable source of chemical products, including different types of biofuels and by-products. Algae yield about 20 times more than existing oilseed crops and could provide considerably more biodiesel than other crops while consuming less water and land and thus appear as a promising feedstock for the ideal third generation biofuel.

However, recent analyses have shown that algal biodiesel production requires further refinement to become economically viable, due to the high cost of lipid extraction (Acién et al., 2013). Anaerobic digestion (AD), which transforms biomass into biogas, is another alternative for recovering energy from algal biomass. Golueke et al. (1957) were the first to report on the AD of two microalgae genera: *Chlorella* and *Scenedesmus*. Sialve et al. (2009) found that AD is a suitable method of generating bioenergy from microalgae, while Zamalloa et al. (2012a) observed low conversion efficiency (30%) when digesting *Scenedesmus obliquus*. González-Fernández et al. (2013) reported a 9.4% biodegradability in a mesophilic continuously stirred tank reactor (CSTR) for untreated *Scenedesmus* spp. Mahdy et al. (2014) found raw *Chlorella vulgaris* to be more biodegradable (50%) than the results of other earlier studies (Mendez et al., 2013). *Scenedesmus* and *Chlorella* have similar rigid cellulose-based cell wall that hampers their degradation, although differences in AD have been observed between these two species. Mussgnug et al. (2010) reported lower methane production when digesting *Scenedesmus* than with *Chlorella* while, Frigon et al. (2013) found *Scenedesmus* to give the highest methane yield.

During AD, the initial organic phosphorus and nitrogen biomass constituents are converted into phosphate and ammonium, which can be recycled for different uses such as microalgae cultivation, reducing the costs associated with commercial fertilizers (Christenson et al., 2011). Nutrient-loaded effluent from an anaerobic membrane

bioreactor (AnMBR) has been shown to be a suitable substrate for microalgae cultivation (Viruela et al., 2016). Greses et al. (2018) reported that AD of *Scenedesmus* from AnMBR effluent reached 146 $mLCH_4 \cdot gCOD_{inf}^{-1}$, working at the low solids retention time (SRT) of 35 d under mesophilic conditions in an AnMBR. This wastewater treatment scheme appears to be a promising approach for circular economy models, since energy, nutrients and reclaimed water are recovered (Seco et al., 2018).

However, a few studies have indicated that biogas production during AD is limited owing to the algal sludge resisting biodegradation (Carrere et al., 2016). In addition, the high protein content yields a low C/N ratio (typically below 10), which could disturb long-term anaerobic mono-digestion (Saratale et al., 2018). The lower the C/N ratio, the higher the concentration of the free ammonia nitrogen (FAN) released, which is an important intermediate product that can unsettle and possibly inhibit the process due to the accumulation of volatile fatty acids (VFA) and lower pH.

One alternative to improve microalgae digestibility is co-digestion with other carbon-rich substrates, such as waste paper, food waste and primary and secondary sludge. Compared with other ways of improving microalgae biodegradability, such as the use of pre-treatments, these carbon-rich co-substrates increase the C/N ratio, improving process stability, increasing efficiency and reducing operating costs. Previous studies have demonstrated the efficiency and cost-effectiveness of anaerobic co-digestion (ACoD) to avoid ammonia inhibition (Saratale et al., 2018; Sialve et al., 2009). Additional studies based on batch tests (Olsson et al., 2014; Wang et al., 2013) have shown that sewage sludge is a suitable feedstock for ACoD with microalgae. Particularly, due to its high lipid content (Solé-Bundó et al., 2019), primary sludge shows promise as a co-substrate for enhancing microalgae biodegradability through ACoD, while this strategy of combining primary sludge and microalgae co-digestion would optimize the management of sludge streams in future water resource recovery facilities (WRRF).

Since AD involves a complex network of metabolic interactions between different microorganisms, analysing the composition of microbial communities can help us to understand microalgae co-digestion. For instance, the acclimation capacity of different inocula for degrading microalgae without any pre-treatment (Gonzalez-Fernandez et al., 2018) is an effective strategy. According to 16S rRNA gene analysis, this capacity is

linked to the presence of Firmicutes, Proteobacteria and Chloroflexi phyla in mesophilic digesters (Greses et al., 2018). However, the microbial structures of microalgae-degrading communities may be different with an added co-substrate and no recent studies have assessed this scenario in a continuous system working at high SRT.

Since AD could be an important step in recycling nutrients for algal cultivation and energy recovery at wastewater treatment plants (WWTPs), further research is needed on AD with microalgae and sewage sludge. Although earlier studies have reported on the co-digestion of different raw materials, all were in batch conditions and some were short-term continuous experiments. Few studies have assessed microbial diversity in a co-digestion system, and this would expand our knowledge of the process and identify the key microbiological members of AD. To the current knowledge of the authors of this manuscript, no studies have evaluated the impact of multiple microalgae species in terms of biogas production in a semi-continuous co-digestion system. The aim of this study was therefore to evaluate the long-term feasibility of semi-continuous ACoD of harvested untreated microalgae in municipal wastewater and primary sludge. The study was carried out in an AnMBR coupled to a biomass reservoir to boost methane production under mesophilic conditions, working at high SRT. This paper reports on the performance of a long-term continuous system with physicochemical and microbial characterization carried out to acquire an in-depth understanding of methane production by raw microalgae and primary sludge ACoD.

4.2. MATERIAL AND METHODS

4.2.1. Substrates

Microalgae and primary sludge were used as feeding substrates. Primary sludge from the gravity thickener of the Carraixet WWTP (Alboraya, Spain) was passed through a sieve with a 0.5 mm mesh size and diluted to adjust the chemical oxygen demand (COD) concentration to the desired value (22.8 $gCOD \cdot L^{-1}$) to keep the organic loading rate (OLR) in the AnMBR constant. Microalgae were collected from a membrane photobioreactor (MPBR) pilot plant (see González-Camejo et al. (2017) for an extensive description). The algal biomass was concentrated by filtration in an external cross-flow, ultrafiltration hollow-fibre membrane unit (HF 5.0-43-PM500, PURON® Koch Membrane Systems) before being fed to the anaerobic reactor. This unit has a nominal

pore size of 500 kDa MWCO (Molecular Weight Cut-Off) and an area of 2.1 m^2. Filtration was carried out to increase the COD concentration from 0.5 to 9.6 $gCOD \cdot L^{-1}$ on average and so achieve the desired OLR value. Both substrates were physicochemically characterized and stored at 4 °C for a maximum of 3 weeks.

4.2.2. Experimental set-up

The experimental set-up for the continuous AD trials consisted of two cylindrical methacrylate CSTRs with a total volume of 14 L and a working volume of 9 L (Figure 4.1). The reactor 1 (AnMBR) was connected to an external hollow-fibre ultrafiltration membrane (UHF) module (PURON Koch membrane systems, 0.03 µm pore size) with a surface area of 0.42 m^2, a total volume of 1 L and a working volume of 0.9 L. Sludge was kept homogenized by continuous recirculation through the UHF, where the effluent was obtained by vacuum filtration. The membrane was programmed to operate in three different stages: filtration, backwash and relaxation. A basic filtration-backwash cycle was performed once per day in order to obtain enough permeate to maintain the desired hydraulic retention time (HRT), and relaxation state was performed during the rest of the day. Transmembrane pressure (TMP) was monitored and recorded to evaluate membrane performance.

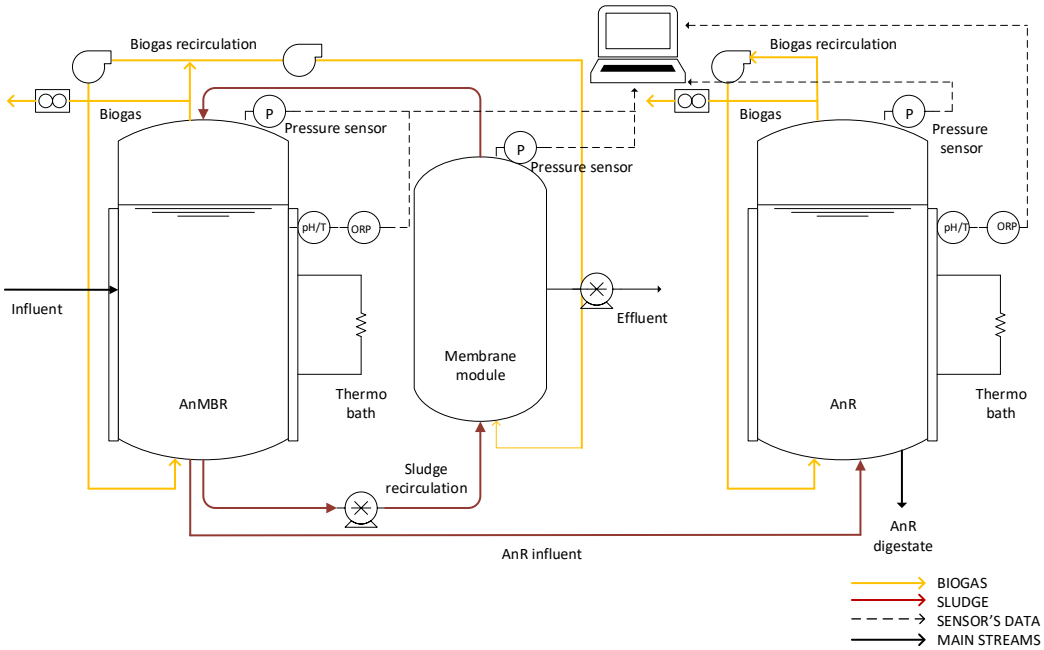

Figure 4.1. Layout of anaerobic co-digestion system showing the anaerobic membrane bioreactor (AnMBR) and the anaerobic reactor (AnR).

The purge stream from the AnMBR was fed to the reactor 2 (AnR), which acted as a biomass reservoir and had the same characteristics as reactor 1 but lacked a membrane (Figure 4.1).

Three biogas blowers were connected in order to allow mixing of both reactors and the membrane module and to control biofilm formation on the membrane. One blower recycled a fraction of the biogas produced in the AnMBR reactor from top to bottom, while another recycled the remaining biogas fraction from the top of the reactor to the bottom of the UHF. The biogas produced in the AnR was recycled from top to bottom of the digester. An MGC 10 milligas counter (Ritter) was used to measure daily volumetric biogas production. Gas volumes were normalized to standard conditions (1 atm, 0 °C).

The system was sealed to avoid gas leakage and was equipped with pH, redox and temperature electrodes. Headspace pressure was also measured in both reactors. A data acquisition program was used to continuously monitor the process.

4.2.3. Operating conditions

AD was performed for 440 days under mesophilic conditions. Both reactors were kept at 35 °C by a water jacket connected to a thermostatic water bath. Reactor 1 was inoculated with sludge from a lab-scale mesophilic reactor digesting microalgae biomass. Reactor 1 was operated at an OLR of 0.52 $gCOD \cdot L^{-1} \cdot d^{-1}$, an SRT of 100 d and at an HRT of 30 d. These values were based on the results of a previous study evaluating SRT impact on microalgae biodegradability (Giménez et al., 2017). Reactor 2 was filled with the daily purge from the AnMBR and was operated at an OLR of 0.15 $gCOD \cdot L^{-1} \cdot d^{-1}$. As the biomass was not recirculated, SRT and HRT were set at 100 days. Reactor 2 thus offers a biomass reservoir and a two-stage system providing a total SRT of 200 d (100 d in the AnMBR plus 100 d in the AnR). The AnMBR was fed with the primary sludge and microalgae mixture in a mass proportion of 62-38% (based on volatile solids (VS) content). This ratio was chosen according to the results of a co-digestion pilot plant simulation (using DESSAS® software) to reproduce the mass proportion produced in a WWTP combining an AnMBR treating settled wastewater followed by an MPBR as a secondary treatment (Seco et al., 2018).

4.2.4. Analytical methods

VS, Total Solids (TS), VFA and Alkalinity (Alk) were measured in duplicate three times per week. Other parameters such as Total and Soluble COD (T-COD and S-COD, respectively), Total Suspended Solids (TSS), Volatile Suspended Solids (VSS), Total Nitrogen (T-N), Ammonium (NH_4-N), Total Phosphorus (T-P), Phosphate (PO_4-P), and Sulphate (SO_4-S) were determined once a week, also in duplicate. All the analyses were performed according to Standard Methods (APHA, 2012).

Biogas samples from the reactor headspace were collected three times a week and methane content was analysed by a Gas Chromatograph fitted with a Flame Ionization Detector (GC-FID, Agilent Technologies), equipped with a thermal conductivity detector and a TRACER column (Teknokroma): 15 m × 0.53 mm × 1 μm. The temperatures of the column and the detector were maintained at 40 °C and 250 °C, respectively. The carrier gas was helium and the flow rate was constant at 5 $mL \cdot min^{-1}$. The sample gas concentration was compared to a standard pure methane gas (99.99%; Air Products Inc.).

The composition of microalgae and primary sludge was analysed by energy-dispersive X-ray spectroscopy (EDS) by searching for the presence of the following elements: C, N, H, and S by means of an XL-30 ESEM (Philips, Netherlands). Substrate samples were attached to the Scanning Electron Microscopy (SEM) stub using silver lacquer. The SEM stub with the sample was then introduced into the XL-30 and the pressure was reduced to 10^{-5} bar, after which the sample surface was visualised and an area was selected for microanalysis. The spot-size value was modified until a dead time of around 30% was achieved.

4.2.5. Biodegradability rate, biomethane potential, methane yield and ammonia concentration calculations.

The anaerobic process efficiency was evaluated in terms of biodegradability percentage, biomethane potential percentage, methane yield (Y^{CH4}) and ammonia concentration using the following equations.

$$\%\ Biodegradability = \frac{CH_4 \sim COD + H_2S \sim COD}{COD_{influent}} \cdot 100 \quad \text{(Eq 4.1)}$$

$$\%\ Biomethane\ potential = \frac{CH_4 \sim COD}{COD_{influent}} \cdot 100 \quad \text{(Eq 4.2)}$$

$$Y^{CH_4} = \frac{CH_4 \sim V}{COD_{influent}}; \frac{L\ CH_4}{g\ COD} \quad \text{(Eq 4.3)}$$

$$[NH_3] = \frac{TAN}{1+\frac{10^{-pH}}{10^{-(0.09018+\frac{2728.92}{T})}}}; \frac{mg}{L} \quad \text{(Eq. 4.4)}$$

where $COD_{influent}$ ($gCOD \cdot d^{-1}$) is the COD of the influent; CH_4~COD is the COD of the produced methane (biogas methane and methane dissolved in the effluent) ($gCOD \cdot d^{-1}$); H_2S~COD is the COD consumed by sulphate reducing bacteria (SRB) for sulphate reduction ($gCOD \cdot d^{-1}$), TAN is the total ammonia nitrogen in the reactor ($mg \cdot L^{-1}$), T is the temperature expressed in Kelvin and CH_4~ V is the volume of methane produced (biogas methane plus dissolved methane in the effluent) at 1 atm and 0 ºC (L).

4.2.6. Microbial population analysis

Inoculum and digestate samples were pelleted after centrifuging 1 mL of sludge at 4000 x g and stored at -20 ºC until nucleic acid extraction procedures. Besides the inoculum, three samples were collected from the AnMBR and AnR after 286, 323 and 400 days of operation. All these samples correspond to the pseudo-steady state period determined when COD, TS and biogas production showed no variation for 154 d and were therefore considered biological replicates. A negative control with sterilized and nuclease-free deionized water was also included at the beginning of the DNA extraction procedure. Following the protocol described by Zamorano-López et al. (2019) the nucleic acid material was extracted from all the samples and used for 16S rRNA amplicon sequencing. Libraries targeting the v3-4 region were sequenced in an Illumina MiSeq in a 2x300 bp paired-end run (Illumina, USA). The resulting raw sequences were deposited on the NCBI SRA depository under Bioproject PRJNA434206 (biosamples SAMN11567546, SAMN11567547, SAMN11567548 from AnMBR; SAMN11567570, SAMN11567571 and SAMN11567572 from AnR; besides inoculum SAMN11567595 and negative control SAMN11567565).

Downstream amplicon sequencing analysis was performed according to a previous study (Zamorano-López et al. 2019) in Quantitative Insights into Microbial Ecology (QIIME) pipelines and R-Studio software plus *vegan* and *mixOmics* packages. The resulting filtered sequences were clustered into Operational Taxonomic Units defined at 3% dissimilarity ($OTU_{0.97}$). Alpha diversity analysis was performed through a Simpson

Evenness Estimator and the number of observed $OTU_{0.97}$. Beta-diversity analysis through principal co-ordinate analysis (PCoA) of the weighted unifrac distance matrix was performed to explore the microbial community structure changes between the inoculum, the pseudo-steady AnMBR and AnR samples, as well as the negative control.

4.3. RESULTS AND DISCUSSION

4.3.1. Source characterization

Harvested raw microalgae and primary sludge were used as feeding substrates during ACoD. The average characteristics of feedstock are shown in Table 4.1. Since an optimized C/N ratio is important for substrate degradation (Yen and Brune, 2007), the elemental composition of both feeding substrates was also determined. Microalgae composition (47.69% C, 9.27% N, 0.61% S, 7.41% H) led to a low C/N ratio of 5.15, as expected, due to its high protein content. Nevertheless, primary sludge composition (40.01% C, 4.42% N, 1.47% S, 6.23% H) also resulted in a low (9.06) C/N ratio. The C/N ratio for the feeding mixture was not that recommended for an AD process, according to previous studies (Sialve et al., 2009; Yen and Brune, 2007).

Table 4.1. Influent composition (mean ± standard deviation values).

Parameter	Unit	Microalgae	Sludge
COD	$mgCOD \cdot L^{-1}$	9739 ± 187	22818 ± 296
TSS	$mgTSS \cdot L^{-1}$	6242 ± 340	14574 ± 623
VFA	$mgCH_3COOH \cdot L^{-1}$	103 ± 68	1126 ± 54
Alk	$mgCaCO_3 \cdot L^{-1}$	310 ± 96	316 ± 157
T-N	$mgN \cdot L^{-1}$	480 ± 85	382 ± 189
NH_4-N	$mgN \cdot L^{-1}$	33 ± 15	111 ± 35
PO_4-P	$mgP \cdot L^{-1}$	4.4 ± 3.4	66 ± 11
T-P	$mgP \cdot L^{-1}$	81 ± 15	175 ± 21
SO_4-S	$mgS \cdot L^{-1}$	92 ± 53	22 ± 14

COD: chemical oxygen demand; TSS: total suspended solids; VFA: volatile fatty acids; Alk: alkalinity; TN: total nitrogen; NH_4-N: ammonium, PO_4-P: phosphate; TP: total phosphorus; SO_4-S: sulphate

Microscopic examination showed that from Days 1 to 60, wastewater microalgae were mainly composed of *Scenedesmus* spp. (>99% of the total eukaryotic cells); from Days 61 to 210, the culture was dominated by *Scenedesmus* spp. and *Chlorella* spp. in different proportions, and from Day 211 to the end of the period, the culture was dominated by *Chlorella* spp. This shift in microalgae species will be discussed in Section 4.3.2.

4.3.2. Anaerobic co-digestion

The harvested microalgae and primary sludge were co-digested under mesophilic conditions in two interconnected anaerobic digesters. Biological process performance was evaluated by monitoring different parameters such as methane production, TS, VS and nutrients concentrations in both reactors (see Figure 4.2). As seen in Figure 4.2b, from Days 71 to 120 biogas production dropped drastically in the AnMBR due to an operating problem related with a gas leakage. After this was solved, methane production rose again and the system rapidly recovered stability. The pseudo-steady state of the sludge and permeate effluents was achieved after 286 days, as seen by TS evolution (Figure 4.2a) and methane production (Figure 4.2b) and was maintained for 154 days. Table 4.2 gives the results obtained in each reactor in the pseudo-steady state. During the last 154 d, the cumulative average biogas production from the system was 1.94 $L \cdot d^{-1}$, where 1.87 $L \cdot d^{-1}$ of this biogas (96.4%) was produced from the AnMBR and the rest (0.07 $L \cdot d^{-1}$) from the AnR. Weekly methane production was therefore much higher in the AnMBR reactor (Figure 4.2b), as expected, since most of the degradation of the feeding substrates occurs in the AnMBR. However, the biogas methane content was similar in both reactors, around 68% (Figure 4.2b), suggesting the presence of an efficient methanogenic population in both AnMBR and AnR. This will be discussed later in the microbial analysis in Section 4.3.3.

The mesophilic AnMBR reached a methane yield of 256 $mLCH_4 \cdot gCOD_{inf}^{-1}$ (435 $mLCH_4 \cdot gSV_{inf}^{-1}$) due to the high hydrolytic activity of the system, which corresponds to an average biomethane potential of 72%. Total biodegradability achieved in the system was around 73%, only 1% of the total biodegradable COD being consumed by SRB. As previous batch assays had shown that primary sludge mono-digestion yielded 64% biodegradability (data not shown), the biodegradability increase up to 73% is related to the synergetic effect of co-digesting primary sludge and microalgae in the AnMBR.

This value is also higher than the findings of previous studies on microalgae digestion. A biodegradability of 9.4% was achieved in a CSTR for untreated *Scenedesmus* spp. (González-Fernández et al., 2013). Greses et al. (2018) reported a methane yield of 35.7 $mLCH_4 \cdot gCOD_{inf}^{-1}$ (11.9% of biodegradability) when digesting *Scenedesmus* microalgae in an CSTR operating at an SRT of 50 days, but a yield of 148.5 $mLCH_4 \cdot gCOD_{inf}^{-1}$ when digesting *Scenedesmus* microalgae in an AnMBR system operating at an SRT of 70 days. Passos et al. (2014) reported 120 $mLCH_4 \cdot gCOD_{inf}^{-1}$ when digesting *Scenedesmus* and *Chlorella* grown in high rate ponds for wastewater treatment and also observed that methane yield was enhanced by 42% with microwave treatment (170 $mLCH_4 \cdot gCOD_{inf}^{-1}$). Although different pretreatments have been studied for microalgae digestion and have shown positive effects on the methane production rate, these treatments are normally costly processes and also have some drawbacks that need to be considered, such as the formation of recalcitrant compounds that could potentially harm the process performance (Mendez et al., 2013). Furthermore, the methane rates achieved in several pretreated microalgae digestion experiments (Magdalena et al., 2018) were lower than the 435 $mLCH_4 \cdot gSV_{inf}^{-1}$ value observed in the present study, demonstrating that ACoD with primary sludge can substantially improve microalgae mono-digestion and increase the methane yield without requiring costly pretreatments. The mix of both substrates accelerated the AD process, as already reported in other studies: Solé-Bundó et al. (2018) observed a threefold increase in methane yield when co-digesting microalgae and primary sludge, compared to pretreated microalgae mono-digestion. The authors reported that the higher the proportion of primary sludge, the higher the methane rate, obtaining the highest methane yield (460 $mLCH_4 \cdot gVS^{-1}$) when adding 75% of primary sludge to pretreated microalgae. This is a little higher than the value observed in the present work, which could be explained by the use of pretreated algae and a higher proportion of primary sludge in Solé-Bundó et al. (2018). The higher the proportion of primary sludge in the AnMBR co-digester the better the result that can be expected. The ACoD proposed here should thus be considered as a less costly strategy for achieving high bioenergy recovery ratios from raw microalgae, since no algae pretreatment was required.

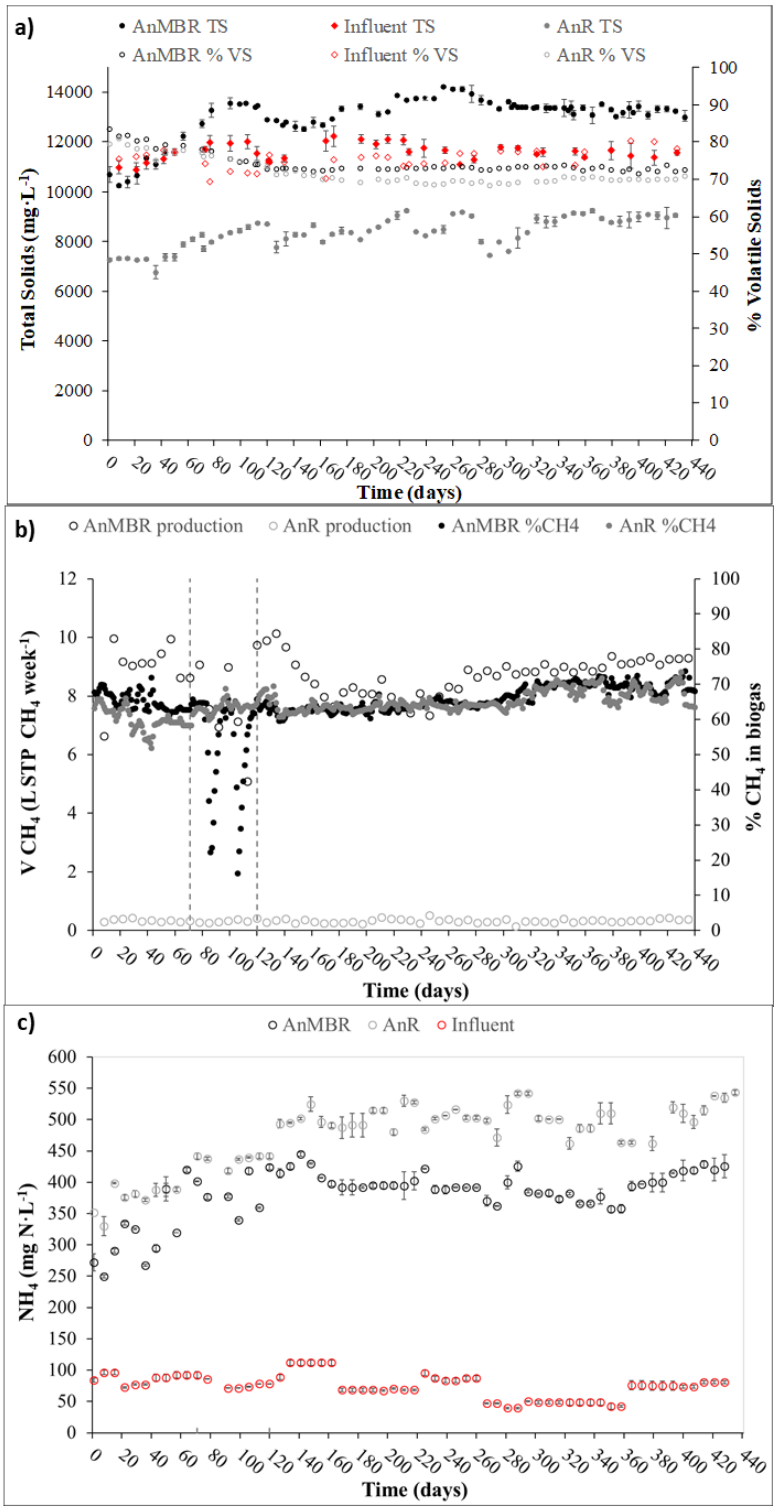

Figure 4.2. Total Solids (TS) and Volatile Solids (VS) evolution over experimental period in the influent stream and both reactors (a), weekly methane production and % CH_4 in biogas in AnMBR and AnR (b) and ammonium concentration in influent and both reactors (c). Error bars represent the standard deviation of replicate measurements.

Table 4.2. Methane production, effluent characterization and removal efficiency from microalgae co-digestion with primary sludge in both reactors (Mean values standard ± deviation). nd: not detected.

	AnMBR	**AnR**
Methane yield ($mLCH_4 \cdot gCOD_{inf}^{-1}$)	256 ± 10	35 ± 10
CH_4 in biogas (%)	68.4 ± 2.5	67.5 ± 2.7
pH	6.9± 0.3	7.2± 0.1
VFA ($mgCH_3COOH \cdot L^{-1}$)	nd	nd
ALK ($mgCaCO_3 \cdot L^{-1}$)	2030 ± 123	2050 ± 115
TP ($mgP \cdot L^{-1}$)	148 ± 19	70 ± 11
PO_4-P ($mgP \cdot L^{-1}$)	28.6 ± 2.2	18.3 ± 0.9
TN ($mgN \cdot L^{-1}$)	857 ± 176	710 ± 96
NH_4-N ($mgN \cdot L^{-1}$)	401 ± 42	503 ± 18
NH_3-N ($mgN \cdot L^{-1}$)	5.15 ± 2.7	12.5 ± 2.5
Biodegradation (%)	73.1 ± 2.6	10.2 ± 2.2
VS removal (%)	70.0 ± 3.3	10.2 ± 3.6
COD removal (%)	70.8 ± 3.4	13.2 ± 2.5

VFA: volatile fatty acids; Alk: alkalinity; TP: total phosphorus; PO_4-P: phosphate; TN: total nitrogen; NH_4-N: ammonium; NH_3-N: ammonia; VS: volatile solids; COD: chemical oxygen demand

This enhanced methane production could theoretically be explained by an optimised C/N ratio due to the addition of primary sludge. As the low C/N ratio of algal sludge is regarded as an important limiting factor to AD, adding primary sludge was expected to improve the C/N ratio to 20-25 (Yen and Brune 2007). As mentioned in Section 4.3.1, a C/N ratio of 5.15 for microalgae and 9.06 for primary sludge was found in the substrates used in this work, but these values were apparently not suitable for AD (Yen and Brune, 2007); however, high biodegradability was achieved in the present work. Primary sludge, mainly formed by colloidal organic matter, is easier to biodegrade and co-digesting it with microalgae seems to have synergy positive effects on substrate biodegradability, as reported by Olsson et al. (2014), who found 23% more methane production when co-digesting sewage sludge with microalgae (63/37% based in VS content) in batch tests. Accordingly, the present results suggest that the presence of primary sludge promoted an efficient microbial community for microalgae degradation, in which syntrophic pathways probably played an important role in degrading the substrate first into intermediate products and later into methane, as will be further discussed in Section 4.3.3.

The mesophilic AnR reached an average biodegradability percentage of 10.2%, which corresponds to a methane yield of 35 $mLCH_4 \cdot gCOD_{inf}^{-1}$, so that the second reactor enabled a 5% improvement in the total methane rate while the system achieved an overall 78% biodegradability percentage.

Regarding microalgae composition, previous studies have reported a dependence on microalgae species for biogas production. In the present study a shift of microalgae species from *Scenedesmus* to *Chlorella* was assessed before achieving a pseudo-steady state: up to Day 210 the culture was dominated entirely by *Scenedesmus* or both *Scenedesmus* and *Chlorella* and from Day 211 to the end of the period it was dominated by *Chlorella* spp. (see Section 4.3.1). Although several studies (Frigon et al., 2013; Mussgnug et al., 2010) have reported a difference in *Chlorella* or *Scenedesmus* anaerobic biodegradability, no differences in terms of biogas production (Figure 4.2b) were found. Several factors, such as culture medium and growth conditions, could have had a significant impact on the specific methane yield. Moreover, the other studies (Frigon et al., 2013; Mussgnug et al., 2010) focused on microalgae mono-digestion. Then, no differences in methane yield were found when co-digesting wastewater-grown *Chlorella* or *Scenedesmus* with primary sludge in an AnMBR, which proved to be a resilient system that would have advantages if the process were to be scaled up.

Process stability in the reactor was evaluated during the whole period by means of pH, VFA, nutrient concentrations and alkalinity. pH evolution was continuously monitored and showed values in the range of 6.8-7.1, so that no pH adjustment was required. VFA did not accumulate in the effluent due to the high alkalinity (Table 4.2) during the whole period. In spite of the high VFA concentrations in the digester influent stream (Table 4.1), due to the high VFA concentration in the primary sludge, the average ratio VFA alkalinity / total alkalinity was kept around 0.01. This value is lower than that maximum value reported in the literature (0.4) for the process to function well and avoids methanogenic reaction being the rate-limiting step (Marti et al., 2008). Total alkalinity should be above 2 $gCaCO_3 \cdot L^{-1}$ to maintain a stable process. In both digesters the total alkalinity was higher than 2 $gCaCO_3 \cdot L^{-1}$ (Table 4.2) indicating stable conditions.

With regard to nutrient concentrations in the AnMBR effluent stream, around 400 $mgNH_4\text{-}N \cdot L^{-1}$ (Figure 4.2c) and 29 $mgPO_4\text{-}P \cdot L^{-1}$ were released from the algae and

sludge ACoD. As a result of the mineralization process, the effluent ammonium concentration was higher than the feeding stream concentration. However, the phosphate concentration was lower (Table 4.1). The fact that the free ammonia concentration (Table 4.2) remained below the typical inhibition concentrations (80 $mg \cdot L^{-1}$) reported in other studies (Garcia et al., 2009) should be noted. The nutrient-rich permeate obtained through microalgae and primary sludge ACoD could be recycled for different purposes, such as algae cultivation in WWTPs (Viruela et al., 2016) or to replace inorganic fertilizers (Zamalloa et al., 2012b). For instance, ammonium could be recovered as ammonium sulphate using ion exchange with zeolites, absorption-desorption or synthetic resins or membrane contactors. Seco et al. (2018) reported an 83% ammonium recovery efficiency in the form of ammonium sulphate while using membrane contactors. Regarding phosphate concentration, the low values observed in the effluent (29 $mgPO_4\text{-}P \cdot L^{-1}$) suggest that a chemical precipitation took place in the reactor and that phosphorus mineralization should be considered and further evaluated (data not shown). Martí et al., (2017) identified the anaerobic digester as a "hot spot" of uncontrolled P precipitation and proposed some alternatives, such as an elutriation step before the AD. Then, research should be addressed to avoid uncontrolled precipitation in this system. Besides that, the digestate had high total nitrogen and phosphorus contents (860 $mgN \cdot L^{-1}$ and 151 $mgP \cdot L^{-1}$ respectively). These nutrient concentrations in the biosolids fraction suggest it could be used in agriculture, as applying biosolids to land has now become popular due to their potential for recycling valuable components such as organic matter, N, P, and other plant nutrients, especially for soils deficient in organic matter (Sharma et al., 2017). In this regard, the present results appear to be a promising strategy for benefitting from the nutrient-rich permeate and biosolids generated in the AnMBR during bioenergy production. It is noteworthy that the AnR digestate also presented a high nutrient concentration, as can be seen in Table 4.2 (around 500 $mgNH_4\text{-}N \cdot L^{-1}$ and 18 $mgPO_4\text{-}P \cdot L^{-1}$).

The ACoD of raw microalgae and primary sludge under mesophilic conditions in an AnMBR thus gave higher methane yields than microalgae or primary sludge mono-digestion. Disruption of the *Scenedesmus* and *Chlorella* cell-wall was achieved without any pre-treatment and no differences were found in the performance of both species of microalgae digestion, offering a robust system for future full-scale implementation.

Wastewater-grown microalgae cannot be widely considered as the main feedstock for full-scale applications because of their low substrate availability and their high resistance to biodegrade without pre-treatment. On the other hand, large amounts of primary sludge are easily available from WWTP, although it does need further treatment. In this context, co-digesting this sludge with microalgae is a promising approach for future WRRF.

4.3.3. Microbial population analysis during microalgae and primary sludge anaerobic co-digestion

16S rRNA gene amplicon sequencing revealed the different compositions of the inoculum and digestate AnMBR samples. Since the AnR was kept as a biomass reservoir during the whole experimental period, the microbial community was also evaluated in this reactor. The aim was to discover the effect of doubling total SRT (200 d) in the system on the microbial population and explore the stability of the microbial community detected in the AnMBR. The beta-diversity analysis of the samples (Figure 4.3a), including the negative control, revealed that the community structure had shifted during the ACoD. Similar diversity was found in both the AnMBR and AnR with minor differences in the evenness of some groups. The Simpson Evenness Index was higher in the AnMBR than AnR samples (0.019 vs. 0.016). The higher number of species in the AnMBR than in the ANR (3852 vs. 2831 $OTU_{0.97}$) could be due to the presence of an ultrafiltration membrane in the AnMBR, which has been reported to increase microbial community diversity in terms of species richness (Greses et al., 2018). The higher diversity could also be explained by the intrinsic microbial diversity of both the microalgae and primary sludge substrates fed to the AnMBR. Accordingly, the species diversity and evenness in the AnR would more accurately reveal the steady community structure of the system, since the AnR's SRT was assumed to be twice that of the AnMBR (200 d).

Figure 4.3b shows the changes in $OTU_{0.97}$ composition identified in the system performance. Different members of Chloroflexi, Proteobacteria, Caldiserica, Firmicutes, Cloacimonetes, Bacteroidetes, Thermotogae and Synergistetes phyla (Bacteria domain) were enhanced during ACoD of microalgae and primary sludge. Among them, Chloroflexi and Proteobacteria were the most abundant, with relative abundances of 17.6% (AnMBR), 18.2% (AnR) and 20.6% (AnMBR), 15.9% (AnR), correspondingly (Table 4.3). Methanogens were established in the system, as revealed by the presence of the Euryarchaeota phylum (Archaea domain).

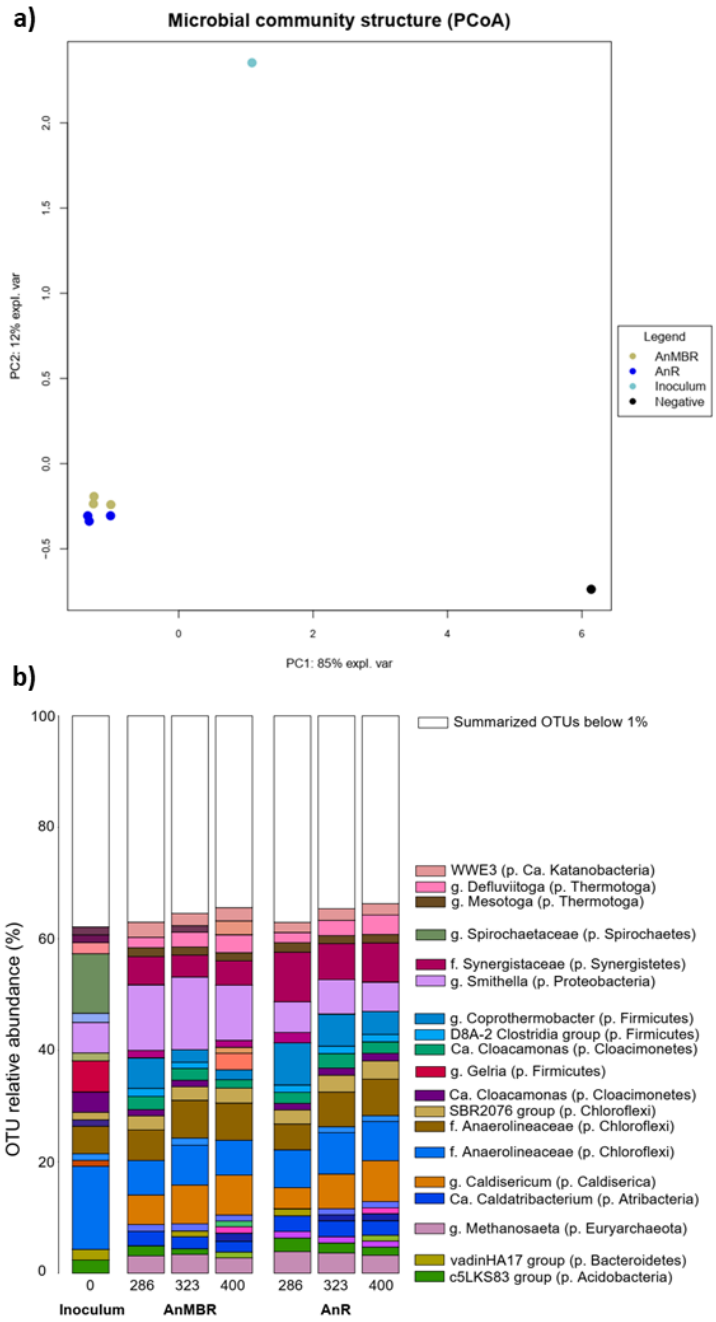

Figure 4.3. a) Principal co-ordinate analysis (PCoA) revealing the microbial structure of the system. Ordination was calculated from the weighted unifrac distance matrix. The two first components were chosen to represent the differences between AnMBR, AnR, inoculum and negative samples analysed in the 16S rRNA gene amplicon sequencing approach. b) Dominant $OTU_{0.97}$ relative abundances barplots (1% relative abundance threshold). The taxonomic level assigned to each $OTU_{0.97}$ is indicated as follows: phylum (p.), family (f.) and genus (g.).

Table 4.3. Mean and standard deviation values of the dominant $OTU_{0.97}$ found in the AnMBR and in the AnR (n=3) plus the composition of the inoculum sample analysed by 16S rRNA gene sequencing.

Taxonomy assignment	Inoculum	AnMBR	AnR
Below 1% relative abundance groups (summarized)	37.9	35.6±1.3	35.1±1.7
d. Bacteria; p. Proteobacteria; c. Deltaproteobacteria; o. Syntrophobacterales; f. Syntrophaceae; g. *Smithella*	5.5	11.6±1.6	5.6±0.5
d. Bacteria; p. Chloroflexi; c. Anaerolineae; o. Anaerolineales; f. Anaerolineaceae	14.9	6.5±0.5	7.1±0.3
d. Bacteria; p. Caldiserica; c. Caldiserica; o. Caldisericales; f. Caldisericaceae; g. *Caldisericum*	0.0	6.5±1.0	5.8±1.8
d. Bacteria; p. Chloroflexi; c. Anaerolineae; o. Anaerolineales; f. Anaerolineaceae; uncultured genus	4.9	6.3±0.7	5.8±1.0
d. Bacteria; p. Synergistetes; c. Synergistia; o. Synergistales; f. Synergistaceae; uncultured genus	0.53	4.4±0.6	7.5±1.3
d. Bacteria; p. Firmicutes; c. Clostridia; o. Thermoanaerobacterales; f. Thermodesulfobiaceae; g. *Coprothermobacter*	n.d.	3.2±2.0	5.8±1.7
d. Archaea; p. Euryarchaeota; c. Methanomicrobia; o. Methanosarcinales; f. Methanosaetaceae; g. *Methanosaeta*	0.4	3.1±0.3	3.6±0.3
d. Bacteria; p. Thermotogae; c. Thermotogae; o. Petrotogales; f. Petrotogaceae; g. *Defluviitoga*	n.d.	2.6±0.7	2.7±0.8
d. Bacteria; p. Chloroflexi; SBR2076 group	1.4	2.6±0.1	2.9±0.4
d. Bacteria; p. Cloacimonetes; Candidatus *Cloacamonas*	4.4	2.0±0.5	2.2±0.3
d. Bacteria; p. Thermotogae; c. Thermotogae; o. Kosmotogales; f. Kosmotogaceae; g. *Mesotoga*	0.2	1.5±0.1	1.6±0.1
d. Bacteria; p. Bacteroidetes; c. Sphingobacteriia; o. Sphingobacteriales; f. Lentimicrobiaceae; uncultured genus	1.0	1.2±0.2	1.0±0.2
d. Bacteria; p. Bacteroidetes; c. Bacteroidia; o. Bacteroidales; f. Rikenellaceae; S50 wastewater-sludge group	0.0	0.7±0.4	0.6±0.3

Non-detected $OTU_{0.97}$ (n.d.). The taxonomic level assigned to each $OTU_{0.97}$ is indicated as follows: phylum (p.), class (c.), order (o.), family (f.) and genus (g.).

The degradation of both co-substrates resulted in a dominance of potential saccharolytic Anaerolineaceae members (phylum Chloroflexi). The inoculum and both reactors shared an $OTU_{0.97}$ related to this family, although changes in its relative abundance were detected. The Anaerolineaceae percentage decreased from 14.9% in the inoculum to 6.5% and 7.1% in the AnMBR and the AnR, respectively. The remarkable dominance of this $OTU_{0.97}$ in the inoculum is related to its previous acclimation to microalgae degradation. Anaerolineaceae are widely reported in mesophilic anaerobic systems and have been likewise detected in recent studies exploring microbial communities during microalgae degradation (Gonzalez-Fernandez et al., 2018; Greses et al., 2018). However, the addition of an extra substrate like primary sludge promoted different groups in the system, yielding a different community in terms of relative abundance distribution. The presence of Anaerolineaceae members in both AnMBR and AnR suggests their importance during saccharolytic fermentation from microalgae and primary sludge.

Hydrolytic microorganisms from other phyla were observed in both reactors besides Chloroflexi saccharolytic fermenters. Members of Thermotogae, a bacterial group capable of sustaining growth over a remarkably wide range of temperature and various simple sugars (e.g., glucose) and complex polysaccharides (e.g., xylan and starch) (De Vos et al., 2009), were observed. Thermotogae includes a mesophilic genus, *Mesotoga*, which has been suggested to be a relevant member during AD of sludge (Lee et al., 2018). The $OTU_{0.97}$ related to *Mesotoga* was detected around 1.6% relative abundance values in both AnMBR and AnR. This *Mesotoga* could be involved in sugar oxidizing processes, including cellobiose and xylose, to produce acetate, sulphide and carbon dioxide, as reported in Lee et al. (2018). Another member of this phylum was found in the present work (*Defluviitoga* genus) at values around 2.6% in both AnMBR and AnR. *Defluviitoga* are able to degrade a large diversity of monosaccharides, disaccharides and polysaccharides, including cellulose and xylan, leading to acetate, ethanol and propionate production (Maus et al., 2016). Finally, members of phylum Bacteroidetes (family Lentimicrobiaceae) were found at a relative abundance of 1.2% and 1.0% in the AnMBR and AnR, respectively. Lentimicrobiaceae contains a strictly anaerobic mesophilic bacteria able to degrade complex polysaccharides such as starch. All the groups mentioned in this paragraph could play an important role in microalgae ACoD, since

similar studies have reported the presence of cellulose in both microalgae and primary sludge (Solé-Bundó et al., 2019).

A remarkable presence of *Smithella* (Deltaproteobacteria) was detected during pseudo-steady state conditions in the AnMBR (11.6%) and the AnR (5.6%). This genus was also observed in the inoculum but at a lower abundance (5.5%). *Smithella* is involved in the conversion of propionate into acetate, a syntrophic pathway known as the "Smithella-pathway" (Leng et al., 2018). This metabolic process can enhance methane production in ACoD as it provides acetate to methanogens. Indeed, together with *Smithella*, the acetoclastic methanogen *Methanosaeta* was observed in both tanks at similar relative abundance values of 3.1% and 3.6% (AnMBR and AnR, respectively). Both microorganisms have been described as common syntrophic partners during methane production and identified as active members of full-scale anaerobic digesters (Mei et al., 2016). The presence of *Smithella* and *Methanosaeta* thus explains the production of methane-riched biogas (68.4% and 67.3% content in the AnMBR and AnR systems, respectively) during microalgae and primary sludge co-digestion. This syntrophic relationship suggests the importance of the synergy obtained by adding an extra carbon source during microalgae co-digestion, such as primary sludge.

Another dominant $OTU_{0.97}$ detected was assigned to *Caldisericum* (phylum Caldiserica). This genus belongs to a poorly described phylum, although recent studies have also detected it in systems with high SRT such as the Up-flow Anaerobic Sludge Bed (UASB) reactors (Chen et al., 2017). This suggests that the role of *Caldisericum* in microalgae and primary sludge ACoD could be related to the high SRT in the reactor. However, further research with complementary approaches to 16S rRNA gene amplicon sequencing, such as proteomics (Heyer et al., 2015) is needed to elucidate its metabolic implications during microalgae co-digestion.

Regarding proteolytic microorganisms, members of Synergistaceae family (phylum Synergistetes) reached high relative abundance in the AnMBR (4.4%) and even higher values in the AnR (7.5%). Although no taxonomic assignment below family level was obtained after $OTU_{0.97}$ clustering, the role of this group in releasing acetate, propionate and butyrate from peptides is commonly known (Jumas-Bilak et al., 2014). Thus, Synergistaceae are suggested as relevant donors of methanogenic substrates in the present

study. Another $OTU_{0.97}$ detected was *Coprothermobacter* (phylum Firmicutes), which is more abundant in thermophilic anaerobic digesters but it has been also isolated from mesophilic systems treating protein-rich wastewater (Etchebehere et al., 1998), indicating that they can tolerate lower temperatures. The high protein content of microalgae (around 60% (Sialve et al., 2009)) could explain the growth of this genus in the system. Moreover, *Candidatus Cloacamonas* genus (phylum Cloacimonetes) a syntrophic bacterium capable of degrading propionate and amino acids, remained in the system at relative abundances of 3% in the AnMBR and 3.5% in the AnR. Proteolytic microorganisms thus play an important role in the co-digestion system.

Rikenecellaceae (Bacteroidetes phylum), a bacteria group capable of degrading amino acids in syntrophic association with hydrogenotrophic methanogens, reached a relative abundance of 0.7% in the AnMBR and 0.6% in the AnR. The release of hydrogen into the system can promote syntrophic-acetate oxidation, enhancing methane production as a result of the higher acetate release in the reactor. According to Leng et al. (2018), the maintenance of a good flux of hydrogen release and consumption during fermentation and hydrogenotrophic methanogenesis can facilitate the production of acetate, which can then be reduced to methane. Hence, the presence of these hydrogen-producing amino acid fermenters related to Bacteroidetes could positively influence microalgae co-digestion, despite their low relative abundances. However, further research considering microbial absolute abundance measurements, with reliance on their activity levels based on RNA-sequencing, besides tracking their metabolism implications (through innovative approaches like proteomics and protein-stable isotope probing) could elucidate the importance of both dominant and minor microbial groups in terms of biogas production.

As shown in Table 4.2, the free ammonia concentration in both AnMBR and AnR remained at low values, although several proteolytic and amino acid degraders were observed in the system (Synergistaceae, *Coprothermobacter*, *Ca. Cloacamonas and* Rikenecellaceae). No VFA accumulation was observed during pseudo-steady state period. The results indicate an absence of FAN inhibition, highlighting the favourable effect of adding primary sludge to improve microalgae digestion.

In conclusion, microalgae and primary sludge hydrolysis and fermentation was performed by polysaccharide-degrading bacteria such as Anaerolineaceae, Thermotogae and

Lentimicrobiaceae, followed by protein disruption by members of Synergistetes phylum besides further fermentation of aminoacids by Rikenecellaceae and *Ca. Cloacamonas*. Syntrophobacterales, mostly composed of syntrophic microorganisms such as *Smithella*, played an important role in microalgae co-digestion, oxidizing key intermediates, such as propionate, providing acetate to methanogens. The presence of methanogens archaea clearly corroborates the ACoD system's performance through methane production.

4.4. CONCLUSIONS

Anaerobic co-digestion of primary sludge and raw microalgae is a feasible approach for enhancing methane production avoiding costly pre-treatments.

- 130 $mLCH_4 \cdot d^{-1} \cdot L_{reactor}^{-1}$ were produced at SRT of 100 d in a mesophilic AnMBR, yielding higher biodegradability (73%) than that achieved by microalgae mono-digestion.
- No ammonia inhibition was detected at working conditions (~5 $mgNH_3\text{-}N \cdot L^{-1}$).
- An enriched fermentative microbial community was found, Chloroflexi and Proteobacteria being the most abundant phyla.
- Bio-nutrients released from anaerobic co-digestion could be reused for microalgae cultivation or agricultural applications.
- Economic assessment of primary sludge and microalgae co-digestion would be required previous large scale implementation of the technology.

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Supplementary material Chapter IV

Table 4.S1. Mean and standard deviation values of the phyla relative abundances in the inoculum, AnMBR and AnR samples.

		Inoculum	AnMBR	AnR
ARC	***Euryarchaeota***	**0.9**	**3.7±0.4**	**4.5±0.4**
ARC	*WSA2*	0.0	0.1±0	0.1±0
BAC	*Acidobacteria*	2.8	1.6±0.6	2.1±0.4
BAC	*Actinobacteria*	1.2	2.2±0.4	2.5±0.1
BAC	*Aminicenantes*	0.6	0.3±0.1	1.1±0
BAC	*Armatimonadetes*	0.7	0.9±0.2	0.9±0.1
BAC	*Atribacteria*	0.6	2.3±0.3	2.8±0.1
BAC	***Bacteroidetes***	**5.0**	**6.8±0.9**	**5.6±0.5**
BAC	*BRC1*	0.1	0.7±0.1	0.6±0.1
BAC	***Caldiserica***	**1.3**	**6.9±1**	**6.1±1.8**
BAC	*Chlamydiae*	0.4	0.2±0.1	0.9±0.2
BAC	*Chlorobi*	0.3	0±0	0±0
BAC	***Chloroflexi***	**25.9**	**17.6±1.3**	**18.2±1.6**
BAC	*Cloacimonetes*	5.3	3.4±0.5	3.9±0.4
BAC	*Fibrobacteres*	0.0	0±0	0±0
BAC	***Firmicutes***	**7.3**	**7.6±1.7**	**10.3±2**
BAC	*Gracilibacteria*	0.0	0.1±0	0±0
BAC	*Hydrogenedentes*	0.5	0.6±0.1	0.7±0.1
BAC	*Lentisphaerae*	0.6	0.3±0.1	0.1±0
BAC	*Microgenomates*	0.0	0.2±0.1	0.2±0
BAC	*Nitrospirae*	0.0	0±0	0±0
BAC	*Omnitrophica*	0.4	0.2±0.1	0.1±0.1
BAC	*Parcubacteria*	0.5	1.8±1.5	0.7±0.3
BAC	*Planctomycetes*	3.8	3.2±0.7	3.2±0.9
BAC	***Proteobacteria***	**21.9**	**20.6±2.3**	**15.9±1.2**
BAC	*Saccharibacteria*	0.2	0.9±0.1	1±0.1
BAC	*Spirochaetae*	10.8	1±0.2	0.7±0.1
BAC	*SR1 (Absconditabacteria)*	0.1	0.1±0.1	0±0
BAC	***Synergistetes***	**0.7**	**4.8±0.7**	**8±1.4**
BAC	*TA06*	0.1	0±0	0.1±0
BAC	***Thermotogae***	**0.2**	**4.5±0.7**	**4.7±0.8**
BAC	*TM6 (Dependentiae)*	0.0	0±0	0.1±0
BAC	***Verrucomicrobia***	**6.8**	**4.2±0.8**	**2.4±0.5**
BAC	*WS2*	0.0	0±0	0±0
BAC	*WS6*	0.1	0.3±0	0.3±0
BAC	*WWE3*	0.0	2.4±0.3	2±0.1

Insights into the biological process performance and microbial diversity during thermophilic anaerobic co-digestion in an anaerobic membrane bioreactor (AnMBR)

5. INSIGHTS INTO THE BIOLOGICAL PROCESS PERFORMANCE AND MICROBIAL DIVERSITY DURING THERMOPHILIC MICROALGAE CO-DIGESTION IN AN ANAEROBIC MEMBRANE BIOREACTOR (AnMBR)

Abstract: Harvested microalgae *Chlorella* spp. and primary sludge were co-digested in a laboratory-scale anaerobic membrane bioreactor (AnMBR) under thermophilic conditions (55 ºC). The system was run for 700 days divided into four experimental phases to determine the influence of the organic loading rate on the process performance and the microbial community. The rise in organic loading rate from 0.17 to 0.5 $gCOD \cdot L^{-1} \cdot d^{-1}$ led to a 35% improvement in methane production. The system reached 69% biodegradability working at 0.5 $gCOD \cdot L^{-1} \cdot d^{-1}$ and a high solids retention time (70 d), indicating the efficient conversion of biomass into biogas through the AnMBR configuration while avoiding possible inhibition by free ammonia. The thermophilic AnMBR provided a stable microbial community dominated by hydrolytic and fermenter members such as *Coprothermobacter*, *Fervidobacterium* and *Hydrothermae*. Syntrophic oxidisers such as *W5* were also key in propionate degradation and produced intermediates for later conversion into methane.

Keywords: Anaerobic membrane bioreactor (AnMBR); co-digestion; microalgae; primary sludge; 16S rRNA gene

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5.1. INTRODUCTION

As the continual growth in the world's population has brought about an increase in the generation of municipal/industrial waste and the consumption of fossil fuels, a paradigm shift from the traditional urban wastewater treatment to a sustainable system is required. Anaerobic membrane bioreactors (AnMBRs) have arisen as a promising technology for wastewater treatment in preference to conventional aerobic treatments due to their many advantages such as the high efficiency in terms of organic matter and suspended solids removal or the high retention of anaerobic microbes since the hydraulic retention time (HRT) and solids retention time (SRT) are uncoupled (Giménez et al., 2011). Microalgae-based wastewater treatment is an especially promising direction for biofuel production through AD, although this is not a new concept, since the idea of producing methane gas from algae was first proposed in the 1950 (Meier, 1955). Since then, many promising results have been obtained for methane production using wastewater-grown microalgae. The high nutrient effluent of an AnMBR system treating urban wastewater has been reported to be a feasible substrate for microalgae cultivation (González-Camejo et al., 2017, Viruela et al., 2018). This means that the combination of a wastewater-grown microalgae cultivation system and AD of produced biomass is a win-win strategy, since it would be feasible to recover both nutrients and energy from wastewater. Indeed, a novel study (Greses et al., 2017a) on the AD of microalgae grown in AnMBR effluent found a methane yield of 98.4 $mLCH_4 \cdot gCOD_{inf}^{-1}$ when digesting *Scenedesmus* spp. in a continuous stirred-tank reactor (CSTR) at 55 ºC.

Operating in thermophilic conditions (50-60 ºC) is known to have several benefits over mesophilic conditions, such as better sanitation of the effluents or better degradation of long chain fatty acids (Kougias et al., 2018). Zamalloa et al. (2012) reported a 1.3-fold higher biogas production rate at 55 ºC than at 35 ºC digesting *Scenedesmus* in a CSTR configuration. Greses et al. (2018) found a threefold rise in methane production digesting *Scenedesmus* spp. in a CSTR changing from mesophilic to thermophilic conditions.

However, thermophilic conditions do have certain drawbacks, such as the energy cost involved in achieving the higher temperature required, while applying high temperatures in AD is associated with the increase of the free ammonia fraction, which inhibits the microorganisms that are involved in the process. This inhibition can be especially

important in the case of feeding high ammonia loads such as microalgae feedstock to the digester. Ammonium (NH_4^+) and free ammonia (NH_3) are produced during nitrogenous matter degradation. Free ammonia nitrogen (FAN) has been suggested to be the most toxic one due to its permeability through the cell membrane, which results in a proton imbalance (Appels et al., 2008). FAN concentration depends on temperature and pH, so that the higher the temperature and pH the higher the FAN concentration. It is difficult to lay down an ammonia inhibition threshold since a wide range of concentrations has been found in the literature that depends not only on pH and temperature, but also on total ammonia concentration (TAN), acclimation, inoculum, and antagonism from other ions and substrates (Chen et al., 2008; Sung et al., 2003). Ammonia toxicity can limit the process performance, reduce methane production and cause the accumulation of volatile fatty acids (VFA), reaching an inhibited pseudo-steady state (Tsapekos et al., 2018) or even complete process failure (Kinnunen et al., 2014; Sung et al., 2003). This means microalgae AD could cause inhibition of anaerobic microbial community, mostly methanogenic archaea (Olsson et al., 2014), so that microbial community evaluation through techniques such as 16rRNA gen amplicon sequencing is a key issue in understanding the biological process.

Anaerobic co-digestion (ACoD) with carbon-rich wastes is an alternative that avoids possible FAN inhibition and improves microalgae digestibility, since these substrates raise the low microalgae C/N ratio (Saratale et al., 2018; Yen and Brune, 2007). Solé-Bundó et al. (2019) observed that co-digesting a mixture of 75% primary sludge and 25% (VS-based) microalgae increased methane production by 65% and had less risk of ammonia toxicity than microalgae mono-digestion in mesophilic conditions. However, Olsson et al. (2014) reported that adding algae to undigested sewage sludge (37/63 %w/w) improved the biochemical methane potential (BMP) significantly in mesophilic conditions but affect BMP negatively in thermophilic conditions and pointed out that microalgae and sewage sludge co-digestion in batch reactors had a synergetic effect at 37 ºC but not at 55 ºC.

ACoD of microalgae biomass and wastewater sludge in mesophilic conditions has therefore shown promising results, although few experiments have been carried out on thermophilic ACoD and all were performed in batch reactors and without positive results (Olsson et al., 2014, Solé-Bundó et al., 2019). Moreover, none of these studies, to the

knowledge of this paper's authors, have assessed the main microorganisms involved in ACoD at thermophilic temperatures and most were based on microalgae cultivated in synthetic media or filtered sewage. The purpose of this work was thus to evaluate the ACoD of harvested wastewater-grown raw microalgae and primary-sludge under thermophilic conditions in a laboratory-scale AnMBR and to study the effect of different organic loading rates (OLR) over the process to enhance methane production. The data on the biological process was supplemented by 16 rRNA gene sequencing to determine the microbial population involved in ACoD and how it is affected by the changes that take place during the reactor process.

5.2. MATERIAL AND METHODS

5.2.1. Experimental set-up and operating conditions

A lab-scale CSTR was used for AD under thermophilic conditions. The biomass was mechanically stirred and temperature was kept at 55 ºC by means of a water jacket. The reactor had a total and working volume of 13 L and 9 L, respectively, and was sealed to ensure anaerobic conditions and avoid biogas leakage. The reactor was inoculated with 9 L of sludge from a lab-scale mesophilic anaerobic digester treating microalgae and primary sludge.

The system was run for 700 days divided into four periods (PI, PII, PIII and PIV). The reactor was fed once a day with a mixture of 62% primary sludge and 38% microalgae based on VS. These proportions were chosen from the results obtained in a previous study (Serna-García et al., 2020). During the start-up (first 45 days), the reactor was operated as a CSTR, at an OLR of 0.22 $gCOD \cdot L^{-1} \cdot d^{-1}$; hence, SRT was equal to HRT (Table 5.1). A ceramic ultrafiltration membrane module (0.1 μm pore size Likuid Nanotek, Spain) was installed on Day 46 and allowed an SRT of 70 days while keeping HRT at 30 days during all periods investigated. The layout of the AnMBR is shown in Figure 5.1. Sludge was continuously recirculated through the membrane in which the permeate was extracted by vacuum filtration. The progressive increase of OLR was studied from Periods PI to PIV (Table 5.1). Pseudo-steady state was considered to be reached when some parameters such as biogas production or solids and nutrient concentrations showed almost no variation for at least around one SRT (70 d) and process was then considered stable and reproducible.

Table 5.1. Operating conditions for the entire experimental period. Mean values ± standard deviation.

	Days	OLR	T^a	Membrane	SRT	HRT
		$gCOD \cdot L^{-1} \cdot d^{-1}$	ºC		d	d
Start-up	0-45	0.22 ± 0.00	59.0 ± 1.0	No	71.6 ± 0.9	71.6 ± 0.9
PI	46-253	0.17 ± 0.01	54.6 ± 1.9	Yes	70.8 ± 1.0	30.1 ± 0.5
PII	254-423	0.30 ± 0.01	54.8 ± 0.9	Yes	70.6 ± 1.2	30.0 ± 0.4
PIII	424-471	0.40 ± 0.01	54.7± 0.9	Yes	70.6 ± 0.6	30.0 ± 0.5
PIV	472-700	0.50 ± 0.03	54.6 ± 0.7	Yes	70.4 ± 0.8	30.1 ± 0.3

OLR: organic loading rate; T^a: temperature; SRT: solids retention time; HRT: hydraulic retention time.

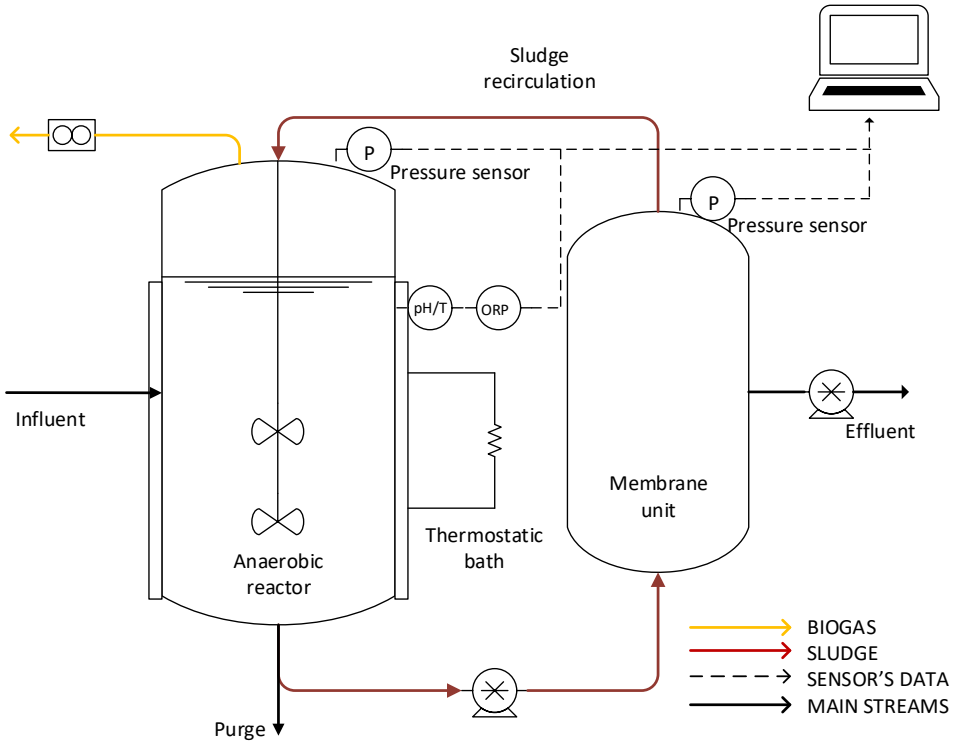

Figure 5.1. Layout of the thermophilic AnMBR.

The AnMBR system was equipped with pH, temperature and redox probes for process monitoring. The headspace reactor and membrane pressure were also monitored and all values were recorded by a custom-written data-logging script in visual basic. Biogas

production was monitored every day through a MilliGas-counter (MGC-1, Ritter, Germany). Litres of biogas produced were normalized to the temperature and pressure conditions of 0 ºC and 1 atm, respectively.

5.2.2. Influent

Primary sludge and microalgae were the substrates used during the four study periods. Microalgae biomass, mainly composed of *Chlorella* spp. (more than 99% of the total eukaryotic cells) was collected from a membrane photobioreactor pilot plant (González-Camejo et al., 2017). Prior to be fed to the AnMBR the wastewater microalgae were concentrated to the desired concentration by filtration in an external cross-flow ultrafiltration hollow-fibre membrane module (HF 5.0-43-PM500, PURON® Koch Membrane Systems, USA), which had a filtration area of 2.1 m^2 and 500 kDa Molecular Weight Cut-Off nominal pore size. Primary sludge was taken from the gravity sludge thickener of the Carraixet's WWTP (Valencia, Spain) and sieved through a 0.5 mm mesh size sieve to discard the large particles, after which the sludge was diluted to obtain the desired COD concentration. Table 5.2 shows the main characteristics of the digester influent stream.

Table 5.2. Influent stream composition (average ± standard deviation, n=2).

	COD	**TS**	**TSS**	**NH_4-N**	**PO_4-P**	**SO_4-S**
	$mg \cdot L^{-1}$	$mg \cdot L^{-1}$	$mg \cdot L^{-1}$	$mg \cdot L^{-1}$	$mg \cdot L^{-1}$	$mg \cdot L^{-1}$
Start-up	15852 ± 150	12036 ± 385	10061 ± 266	99.2 ± 13.2	42.8 ± 1.4	17.7 ± 3.8
PI	5098 ± 213	3888 ± 69	3313 ± 68	46.9 ± 9.6	37.0 ± 6.0	26.9 ± 5.7
PII	9004 ± 168	6933 ± 89	5990 ± 102	71.6 ± 14.3	47.0 ± 12.7	30.4 ± 2.2
PIII	11959 ± 435	9057 ± 122	7640 ± 388	43.6 ± 10.5	26.5 ± 4.2	32.6 ± 3.3
PIV	14945 ± 706	12027 ± 385	9440 ± 291	62.1 ± 23.7	17.3 ± 6.1	48.6 ± 4.7

COD: chemical oxygen demand; TS: total solids; TSS: total suspended solids; NH_4-N: ammonium; PO_4-P: phosphate; SO_4-S^-: sulphate.

5.2.3. Analytical methods

Reactor and substrates samples were regularly analysed to control the biological process. Total Solids (TS), VS, Alkalinity (Alk) and VFA were determined three times a week in duplicate. Other parameters such as Total COD (T-COD), Soluble COD (S-COD), Total Suspended Solids (TSS), Volatile Suspended Solids (VSS), Phosphate (PO_4-P), Total Phosphorus (TP), Sulphate (SO_4-S), Ammonium (NH_4-N) and Total Nitrogen (TN) were measured also in duplicate once a week. All the analyses were based on the guidelines in the Standard Methods (APHA et al., 2012).

The reactor headspace methane content was measured three times per week by a gas chromatograph equipped with a flame ionization detector (GC-FID, Agilent Technologies, USA), and a TRACER column (Teknokroma, Spain): 15 m × 0.53 mm ×1 μm. The temperatures of the detector and the column were kept at 250 and 40 °C, respectively. A flow rate of 5 $mL \cdot min^{-1}$ was used for the carrier gas (helium). The concentration of the sample gas was contrasted with a standard pure gas (99.9995% methane).

5.2.4. Calculations and statistical analysis

NH_3, biodegradability percentage and methane yield were calculated to control the process performance according to equations previously reported in literature (see Serna-García et al. (2020) for an extensive description).

All analytical determinations were performed in duplicate (except for measurements with more than 10% difference, in which the analyses were performed in triplicate) to calculate the average and standard deviation (n=2) shown in graphs and tables.

The results obtained were statistically analysed by SPSS® Statistics 26 software. An analysis of variance (ANOVA) was performed to evaluate the significance of the differences in the mean values. A bivariate Pearson correlation analysis was also performed to know the influence of OLR over different parameters. Differences were considered statistically significant when p-value was below 0.05.

5.2.5. Microbial population analysis

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

A total of 17 samples (Table 5.3) were extracted from the reactor. Samples #1 to #2 were extracted during the start-up and #3 to #4 during PI to monitor the microbial community evolution after start-up. Samples #5 to #17 (excluding #11, #12, #13 and #14) were extracted when pseudo-steady state was achieved (i.e. 3 samples in each of the four study periods) in terms of biogas production, VFA accumulation, nutrients concentration and TS accumulation, and can thus be considered biological replicates. Samples #11, #12, #13 were extracted during PIII before achieving pseudo-steady state. Sample #14 was extracted during an operational problem in period PIV to monitor its effect on the microbial community. Pellets of 1 mL digestate samples were stored in 2 mL cryotubes at −20 °C. The E.Z.N.A DNA Extraction Kit for Soil (Omega-Biotek, USA) was used to extract nucleic acid material from 1 g of biomass, in accordance with the manufacturer's protocol. A Nanodrop 2000 spectrophotometer (Thermo Scientific, USA) was used to determine DNA concentration and purity through the absorbance measured at wavelengths of 260, 230 and 280 nm. To avoid RNA, humic acids or other compounds contamination, only sequences with an A260/280 ratio over 1.8 and an A260/230 ratio between 2.0 and 2.2 were sequenced.

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 805R 5'-GACTACHVGGGTATCTAATCC-3′) (Klindworth et al., 2013). The sequencing run was carried out in a 2×300 base pairs paired-end run using v3 chemistry in a MiSeq Sequencer (FISABIO, Valencia, Spain).

5.2.5.1. Illumina data processing and statistical analysis

The resulting raw sequences were downstream processed to remove chimeras following the *MiSeq_SOP* pipeline (website accession data was 21st June 2019) using open-source *Mothur* software (v.1.42). The taxonomy was assigned according to the v132 release of SILVA database. Alpha diversity was calculated through the evenness index indicators: Shannon-Wiener and Simpson (expressed as inverse Simpson). A principal co-ordinate

analysis (PCoA) based on the Bray-Curtis distances matrix was performed to explore the beta diversity of the different samples collected from the AnMBR. Subsampling was applied to the minimum level of sequences obtained (50977 sequences) for both alpha and beta diversity.

Table 5.3. Sample collection for 16 rRNA gene sequencing.

Period	Sample Number	Day of operation	Comments
Start-up	#1 #2	17 and 36	Before membrane installation
PI	#3 #4	50 and 64	After membrane installation
	#5 #6 #7	232, 238 and 247	Pseudo-steady state
PII	#8 #9 #10	263, 276 and 290	Pseudo-steady state
PIII	#11 #12 #13	466, 470 and 472	Transient state
PIV	#14	552	After operational problem
	#15 #16 #17	650, 662 and 670	Pseudo-steady state

5.3. RESULTS AND DISCUSSION

5.3.1. Effect of increased OLR on reactor performance

The reactor was inoculated with sludge from a mesophilic anaerobic co-digester. Since a thermophilic system is more susceptible to instability than a mesophilic, a low OLR (0.22 $gCOD \cdot L^{-1} \cdot d^{-1}$) was chosen to start-up the lab-scale thermophilic CSTR. Despite the low initial organic load, an accumulation of VFA and NH_3 and a low pH (Figure 5.2) were found during the start-up period, suggesting possible NH_3 inhibition. This parameter showed a maximum value of 29.9 $mgNH_3\text{-}N \cdot L^{-1}$, which is lower than inhibiting concentrations reported in many previous studies (Appels et al., 2008; Yenigün and Demirel, 2013). However, it should be noted that ammonia toxicity relies on several factors such as pH and temperature or inoculum source, and then, an absolute inhibition concentration cannot be quoted.

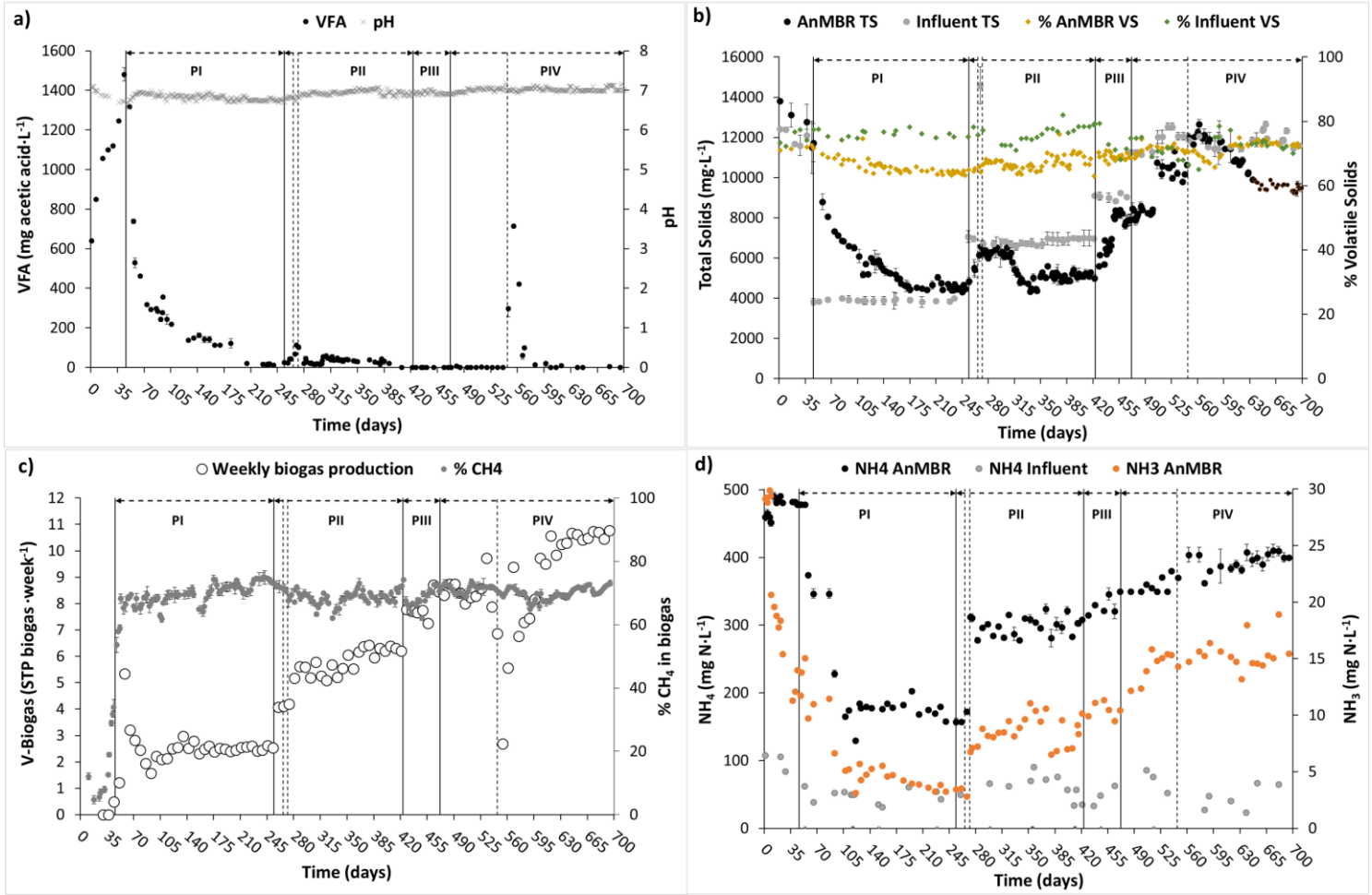

Figure 5.2. Anaerobic membrane bioreactor (AnMBR) pH and volatile fatty acids (VFA) concentration (a), AnMBR and influent stream total solids (TS) concentration and volatile solids (VS) percentage (b), weekly biogas production and methane (CH_4) percentage (c) and ammonium (NH_4) and ammonia (NH_3) concentration (d) during anaerobic co-digestion**.**

Free ammonia concentration is in equilibrium with ammonium ion and its concentration depends on the pH value, which was lower than 7 in this period (Figure 5.2a), achieving the lowest value (i.e., 6.55) of the entire study period. It should also be noted that during the start-up the temperature control was not precise and the temperature was 4 ºC higher than in the other periods (Table 5.1). VFA reached a concentration peak of 1480 mg$CH_3COOH \cdot L^{-1}$ whilst alkalinity reached the lowest value: 454 mg$CaCO_3 \cdot L^{-1}$, showing a VFA alkalinity / total alkalinity ratio (0.7) above the maximum recommended (0.4) for a stabilized anaerobic digester (Zhao et al., 2004). For that reason, a ceramic membrane was installed on Day 46 and a bit lower OLR (0.17 g$COD \cdot L^{-1} \cdot d^{-1}$) was set during PI. The reactor then became an AnMBR and this enabled the dissociation between SRT and HRT (Table 5.1). Besides the previously reported advantages of AnMBR systems (Giménez et al., 2011), recent studies (Greses et al., 2017a, Serna-García et al., 2020) found that operating a mesophilic AnMBR at high SRT promoted retention of microorganisms with a low growth rate, which created a unique microbial community capable of degrading the *Scenedesmus* or *Chlorella* cell-wall. After 130 days of AnMBR operation the effluents achieved a pseudo-steady state and there was no sign of inhibition. Biogas production was 0.37 $L \cdot d^{-1}$ with methane content around 69%. The system reached a methane yield of 176 m$LCH_4 \cdot gCOD_{inf}^{-1}$ (302 m$LCH_4 \cdot gSV_{inf}^{-1}$) resulting in a biodegradability percentage of 51.4%.

Earlier studies (Appels et al., 2008; Kwietniewska et al., 2014) have shown that the higher the OLR, the higher the biogas production rate. Once the system reached pseudo-steady state conditions with no inhibition, a higher OLR (0.30 g$COD \cdot L^{-1} \cdot d^{-1}$) was studied during PII. The higher OLR caused a temporary accumulation of TS (Figure 5.2b) and VFA (Figure 5.2a) at the beginning of PII. Due to an experimental problem, the system was fed with triple OLR for one week (0.65 g$COD \cdot L^{-1} \cdot d^{-1}$), from Day 266 to 272 (shown as two close-dashed lines; Figure 5.2). Even with this abrupt temporary peak of organic load the VFA reached a concentration of 111 mg$CH_3COOH \cdot L^{-1}$ (much lower than during start-up) and alkalinity did not decline. The VFA accumulation along with the higher TS could indicate possible process inhibition. However, biogas production continued to rise (Figure 5.2c) and pH, which directly affects the free ammonia concentration, remained stable, indicating that the system's toxicity levels had not been achieved. These results suggest that the methanogens could only have been partially inhibited or they were able to tolerate

the ammonia concentration present in the system. VFA and TS concentrations decreased after 20 and 30 days, respectively. The steady state in PII was achieved from day 350 onwards, as can be seen in Figure 5.2. This stability was also confirmed by the low VFA (Figure 5.2a) and high alkalinity effluent values (Table 5.4). The TS concentration reached in this period was similar to that achieved during the PI pseudo-steady state, since the process was more efficient during this period in terms of VS removal (Table 5.4). The reactor showed a biogas production of 0.88 $L \cdot d^{-1}$ with a methane content around 69%. The system reached a methane yield of 229 $mLCH_4 \cdot gCOD_{inf}^{-1}$ (380 $mLCH_4 \cdot gSV_{inf}^{-1}$) giving rise to 66.1% biodegradability.

Table 5.4. Anaerobic co-digestion performance. Mean values ± standard deviation (n=2) of the main performance indicators for each period.

	START-UP	**PI**	**PII**	**PIII**	**PIV**
NH_4-N ($mgNH_4$-N·L^{-1})	454± 10	175 ± 8	303 ± 12	319 ± 13	426 ± 42
NH_3-N ($mgNH_3$-N·L^{-1})	20.8 ± 6.9	4.3 ± 0.7	8.9 ± 1.6	10.5 ± 0.8	15.8 ± 1.7
TN ($mgN \cdot L^{-1}$)	772 ± 85	280 ± 25	426 ± 51	538 ± 140	648 ± 164
PO_4-P ($mgPO_4$-P·L^{-1})	13.3 ± 3.9	9.6 ± 1.6	14.0 ± 0.5	13.5 ± 0.7	20.8 ± 0.7
TP ($mgP \cdot L^{-1}$)	95 ± 24	40 ± 12	46 ± 13	52 ± 7	81 ± 12
VFA ($mgCH_3COOH \cdot L^{-1}$)	1070 ± 269	15.2 ± 4.0	24 ± 9.0	n.d.	n.d.
ALK ($mgCaCO_3 \cdot L^{-1}$)	599 ± 208	1017 ± 76	1286 ± 101	1483 ± 161	2030± 110
Methane yield ($mLCH_4 \cdot g\ COD_{inf}^{-1}$)		176 ± 7	229 ± 7	217 ± 7	242 ± 8
% CH_4 in biogas		68.5 ± 6.5	68.9 ± 2.7	69.1 ± 3.2	70.5± 1.8
% Biodegradation		51.4 ± 1.9	66.1 ± 2.0	62.5 ± 3.5	69.2 ± 2.0
% VS removal		53.6 ± 3.4	66.7± 2.9	64.5 ± 5.3	69.2 ± 4.9
% COD removal		53.7 ± 1.8	66.8 ± 3.8	69.2± 3.1	70.3 ± 1.8

NH_4-N: ammonium; NH_3-N: ammonia; TN: total nitrogen; PO_4-P: phosphate; TP: total phosphorus; VFA: volatile fatty acids; Alk: alkalinity; CH_4: methane; VS: volatile solids; COD: chemical oxygen demand; n.d.: not detected

After PII, the OLR was increased first to 0.4 $gCOD \cdot L^{-1} \cdot d^{-1}$ in PIII and later to 0.5 $gCOD \cdot L^{-1} \cdot d^{-1}$ in PIV. The aim in PIII was to allow a progressive OLR increase to avoid possible inhibitions. However, the PIII results could not be determined with certainty, since steady state was not achieved, although TS started to rise with the higher OLR but without either VFA or NH_3 accumulation (Figure 5.2). OLR was thus raised to

0.5 $gCOD \cdot L^{-1} \cdot d^{-1}$ on Day 472. Although TS accumulation was observed, the VFA values did not change and the system seemed about to reach the pseudo-steady state. However, there was an operational problem on Day 547 related with the thermostatic water bath and the temperature rose during 4 hours, up to 70 ºC and then was rearranged recovering 55 ºC. This abrupt temperature change caused a sharp rise in VFA concentration and biogas production dropped considerably (see Figure 5.2). Since temperature is an important factor in microbial activity (Demirel and Scherer, 2008) this 15 ºC increase could have altered the microbial community in the reactor so that the microorganisms were not able to transform the large amount of acetate into methane (see further discussion in Section 5.3.2), suggesting that the methanogenic reaction was the rate-limiting step. After 40 days the system recovered itself without changing any operating condition and biogas production rose to 1.52 $L \cdot d^{-1}$ with a methane content of around 70%. The thermophilic AnMBR showed its best performance during period PIV (Table 5.4) with a methane yield of 242 $mLCH_4 \cdot gCOD_{inf}^{-1}$ (429 $mLCH_4 \cdot gSV_{inf}^{-1}$), which corresponds to a biodegradability percentage of 69%.

Although methane content in biogas was similar in all periods (p-value > 0.05), the ANOVA results obtained clearly show considerably better methane production when the OLR was raised in the thermophilic AnMBR (p-value < 0.05). In fact, a positive Pearson's correlation (0.828) was found between OLR and methane yield when applying a bivariate Pearson correlation analysis. The higher OLR led to a VFA accumulation at the beginning of PI and PII (Figure 5.2a), due to the higher acidogenic bacteria growth rate over methanogenic archaea. The OLR increase also led to a higher NH_3 concentration (p-value < 0.05). Nevertheless, the system always recovered stability and there was no inhibition by overloading during any pseudo-steady state. The positive Pearson's correlation (0.909) between alkalinity and OLR also demonstrates the stability of the system. The threefold OLR rise (from 0.17 to 0.5 $gCOD \cdot L^{-1} \cdot d^{-1}$) improved methane yield by 35%, which differs from the findings of Olsson et al. (2014), who observed a negative effect on methane yield when digesting microalgae and primary sludge in a blend of 37/63% on VS, respectively, in thermophilic batch reactors, compared to microalgae mono-digestion. Nevertheless, the effect of raising OLR on methane yield in the present study is in agreement with a number of studies on microalgae mono-digestion in mesophilic and thermophilic conditions. González-Fernández et al. (2013) found a 15% improvement in

methane yield when OLR was raised by 2.5 in a mesophilic CSTR, and Greses (2017) found a 17% improvement when doubling the OLR in a mesophilic AnMBR. Greses (2017) also obtained a 19% methane yield enhancement when doubling OLR from 0.2 to 0.4 $gCOD \cdot L^{-1} \cdot d^{-1}$ digesting only microalgae in a CSTR operated at 55 ºC. The thermophilic ACoD of primary sludge and microalgae in an AnMBR in the present work thus shows important improvements over thermophilic microalgae mono-digestion in a CSTR. Greses (2017) reported 46.8% biodegradability and a methane yield of 164 $mLCH_4 \cdot gCOD_{inf}^{-1}$ when digesting raw microalgae (*Scenedesmus* spp.) at 0.3 $gCOD \cdot L^{-1} \cdot d^{-1}$ of OLR and 70 d SRT in a thermophilic CSTR. In the present work, with the same OLR and SRT, 66.1% biodegradability was obtained, reaching a methane yield of 229 $mLCH_4 \cdot gCOD_{inf}^{-1}$. Zamorano-López et al. (2019) obtained 38% biodegradability when digesting raw microalgae (*Scenedesmus* spp.*)* at 0.4 $gCOD \cdot L^{-1} \cdot d^{-1}$ of OLR and 50 d of SRT. In the present work, 65% biodegradability was obtained during PIII, working at the same OLR and a slightly higher SRT (70d). The thermophilic co-digestion of microalgae and primary sludge in an AnMBR therefore obtains higher methane production than microalgae mono-digestion. The addition of a more biodegradable substrate with higher lipid content as the primary sludge generates a synergistic effect on microalgae digestion, as will be discussed in Section 5.3.2.

5.3.2. Microbial community characterization in the thermophilic AnMBR

The microbial community involved in the thermophilic ACoD was mainly composed of 98-99% bacteria and 1-2% archaea in all the periods investigated, in agreement with other studies (Klassen et al., 2017; Zamorano-López et al., 2019). Bacterial groups are involved in three of the four key steps of the AD processes, with a high variety of substrate preferences, while archaea only participate in the last AD step with a smaller range of substrate options. The thermophilic AnMBR is richer in terms of observed taxonomic groups than thermophilic microalgae AD in a CSTR (Zamorano-López et al., 2019) or thermophilic full-scale both AD or ACoD (Sundberg et al., 2013). The 16 rRNA gene sequencing provided 1658978 raw sequences, generating 78 phyla (the most dominant are shown in Figure 5.3), 126 classes, 319 orders, 576 families, and 1632 genera. Consequently, high Shannon-Wiener and inverse Simpson indexes were found in all samples (Figure 5.4). The highest diversity was observed in Samples #1 and #2, with inverse Simpson indexes of 17 and 15, respectively, probably associated with the

mesophilic inoculum diversity. Nevertheless, a high diversity was also observed in Periods PI, PII, PIII and PIV. Shannon-Wiener and inverse Simpson estimators showed an average value of 3.4 and 9.7, respectively. Similar diversity was observed between periods, with minor differences in the evenness estimators (Figure 5.4). The beta-diversity analysis of the samples showed that the microbial community composition had shifted after membrane installation (Figure 5.S1 in the supplementary material), as will be discussed in the bacteria and archaea population diversity description (Section 5.3.2.1 and 5.3.2.2, respectively). Although the four periods were similar in diversity, there was a change in the microorganisms' relative abundance when working at different OLR.

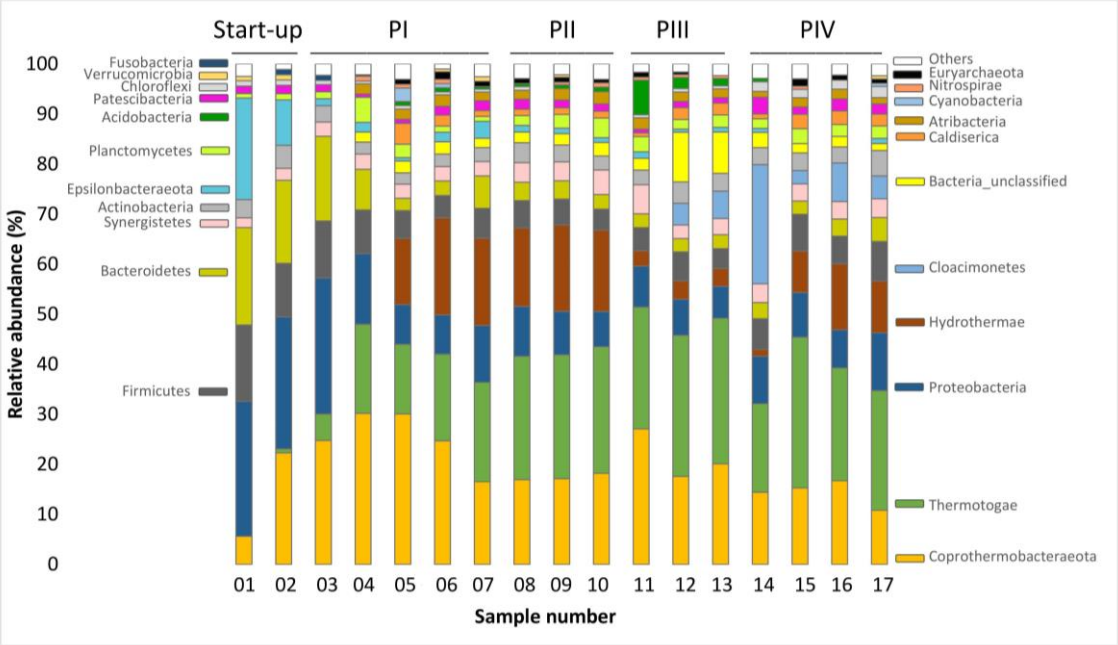

Figure 5.3. Bacterial and archaeal phyla relative abundance (1% relative abundance threshold). The rest of the groups detected are summarized and represented as "Others".

5.3.2.1. Bacteria population

The digestate composition shown in Figure 5.3 highlights the dominance of four phyla during all the study periods: Coprothermobacteraota, Thermotogae, Hydrothermae, and Proteobacteria, followed by Firmicutes, Bacteroidetes, Actinobacteria and Synergistetes.

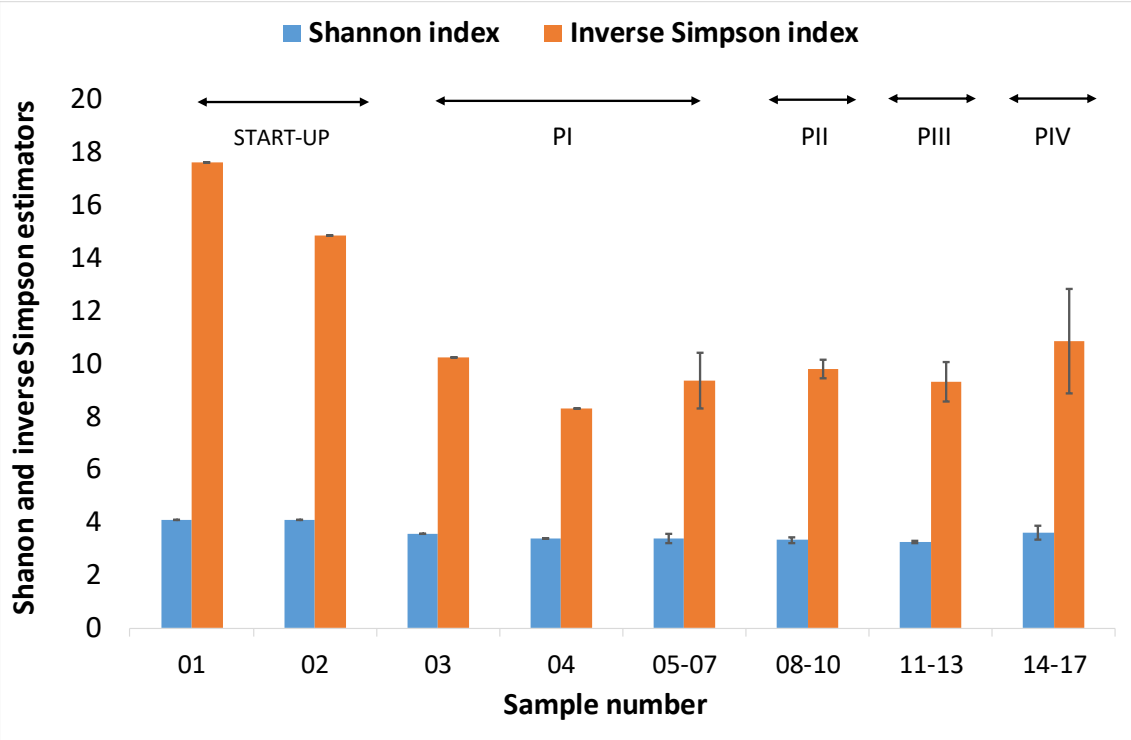

Figure 5.4. Alpha diversity estimator values (Shannon and inverse Simpson indexes) from samples in the start-up and the four periods investigated.

Several differences can be observed from Samples #1 to #4 in terms of dominant bacteria genera (Figure 5.5a). Most of the groups in Samples #1 and #2 were not present after membrane installation, when an adapted microbial community was established and maintained during all the pseudo-steady state periods. For instance, a remarkable group of Epsilonbacteraeota (*Arcobacter* genus) was found in Sample #1 (15 days after start-up) at a relative abundance around 19.7%. Its presence decreased progressively over time (Figure 5.5a) and it was eliminated from the system after Sample #9. Kirkegaard et al. (2017) studied the difference in microbial diversity between primary sludge and thirty-two full-scale anaerobic digesters; *Arcobacter* was one of the most dominant bacterial genera in the primary sludge (one of the co-substrates digested in the present work) and it was hardly detected in anaerobic digesters. Within this genus several pathogenic species have been described for humans and animals, as well as in *Pseudomonas* genus (Shrestha et al., 2018), which was also present in the system only in the first 4 samples. The disappearance over time of these genera indicates the high efficiency of thermophilic systems in pathogens destruction.

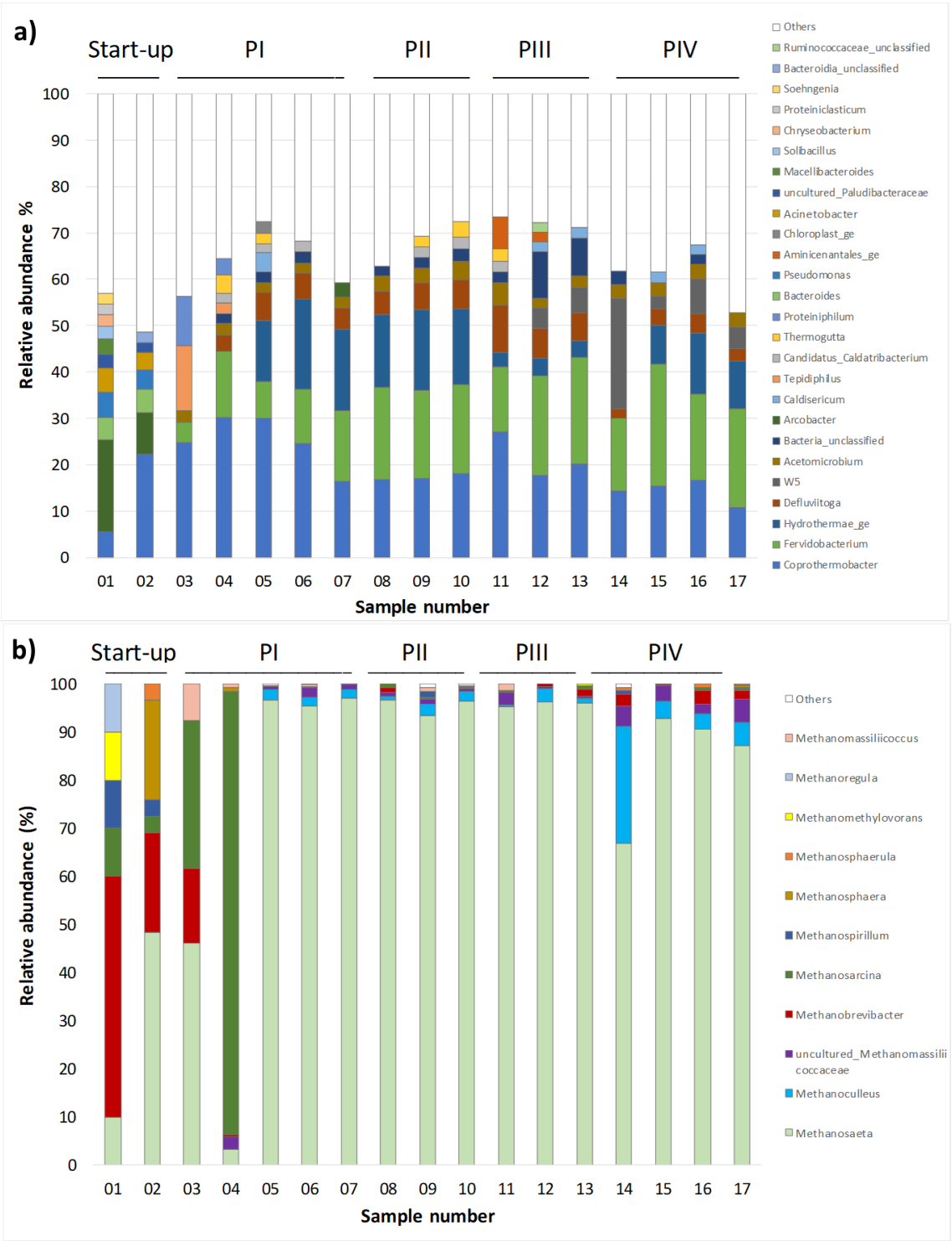

Figure 5.5. Dominant relative abundance barplots (2% relative abundance threshold) of: Bacteria genera-based taxonomic units (a) and Archaea genera-based taxonomic units (b). The rest of the groups detected are summarized and represented in as "Others".

The appearance of *Tepidiphilus* genus in Samples #3 and #4 at a relative abundance of 14 and 2.5%, respectively, should be noted. *Tepidiphilus* is a moderately thermophilic bacterial group, normally present in thermophilic aerobic digesters (Manaia et al., 2003). The presence of this bacterium may be due to the fact that, during the installation of the membrane (5 days before sampling #3), some air could have entered the system. This fact could also have enhanced the presence of *Proteiniphilum* (see Table 5.S1), a facultative anaerobic organism characterised as an acidogenic microorganism producing acetate, propionate and isovalerate (Hahnke et al., 2016). Both organisms disappeared after Sample #4.

Since microalgae was one of the co-substrates, the reactor exhibited a high diversity of hydrolytic and fermentative groups with high proteolytic and cellulolytic activities. Firmicutes, Bacteroidetes and Proteobacteria phyla were abundant (Figure 5.3) during all the periods studied. These phyla are commonly found in thermophilic anaerobic digesters (Greses et al., 2017; Maus et al., 2016) due to their ability to hydrolyse and ferment polysaccharides and proteins.

Coprothermobacter, a member of the novel phylum Coprothermobacteraota (Pavan et al., 2018), was one of the most abundant genera in the AnMBR (Figure 5.5a). The high proteolytic activity of this phylum has been extensively reported (Cagliano et al., 2015; Pavan et al., 2018) and it has been detected in thermophilic anaerobic digesters treating high-protein substrates such as microalgae or cow manure (Cagliano et al., 2015; Greses et al., 2017). This bacterium can also improve protein degradation by establishing a syntrophic relation with hydrogenotrophic archaea, such as *Methanoculleus*, the second most abundant archaea in the AnMBR (as will be explained in Section 5.3.2.2.). Another proteolytic microorganism from phylum Synergistetes (*Acetomicrobium*) was observed during the whole experimental period at a relative abundance of approximately 3% (Table 5.S1in the supplementary material). This genus, recently reclassified inside Synergistetes phylum (Hania et al., 2016), has been described not only as fermenting peptide bacteria but also as syntrophic bacteria (Oosterkamp et al.., 2019).

Besides protein fermenters, hydrolytic microorganisms from phylum Thermotogae were also observed. *Fervidobacterium*, which represents around 80% of the phylum, was not present in the first 2 samples. Accordingly, Tian et al. (2015) also observed that

Fervidobacterium relative abundance raised from 0 to around 28% in 18 days when studying change in the microbial community when inoculating a thermophilic digester with mesophilic sludge. This thermophilic cellulolytic bacterium is able to grow in a large range of organic substrates such as cellulose, glucose, lactose or xylan (Podosokorskaya et al., 2011). Another member of this phylum, *Defluviitoga*, was observed for the first time in Sample #4 and remained during all the periods at a relative abundance of around 5.5% (Table 5.S1 in the supplementary material). The *Defluviitoga* bacteria group is able to grow in a wide range of mono and polysaccharides releasing acetate, ethanol, CO_2 and H_2 (Sundberg et al., 2013). Two other genera found in significant quantities were *Thermogutta* (Planctomycetes phylum) and *Candidatus Caldatribacterium* (Atribacteria phylum) (Figure 5.3), whose function in AD processes is also associated with hydrolysis and fermentation of carbohydrates. Both of them have already been reported in thermophilic microalgae AD (Zamorano-López et al., 2019). These bacteria are thus key elements in degrading polysaccharides, including cellulose and derivatives while they produce hydrogen during microalgae and primary sludge ACoD.

W5 genus rose from below 0.5% to 4-5% abundance (Table 5.S1) when the OLR was 0.4 $gCOD \cdot L^{-1} \cdot d^{-1}$ (from Sample #12). However, its relative abundance increased to 23.8% in Sample #14. *W5* is known to be an anaerobic mesophilic acetogen but it has also been observed in a thermophilic propionate-degrading enrichment culture, where other known syntrophic propionate oxidiser bacteria (SPOB) were hardly found (Dyksma and Gallert, 2019). *W5* is involved in propionate degradation, acetate being one of the products of this degradation. This metabolic process can boost methane yield in ACoD, as it produces acetate that will be used by methanogens as *Methanosaeta* (the predominant archaea during the pseudo-steady state). However, propionate degradation also provides H_2 for hydrogenotrophic methanogens such as *Methanoculleus*. Indeed, a higher presence of this archaea genus (*Methanoculleus*) was found in Sample #14, together with higher *W5* abundance, as will be described in Section 5.3.2.2. This is in agreement with Dyksma and Gallert (2019), who found the highest abundance of *Methanoculleus* when *W5* was the most abundant bacterium. The presence of *W5* together with methanogens working at high OLR (0.4 or 0.5 $gCOD \cdot L^{-1} \cdot d^{-1}$) could explain the high biogas methane content.

Candidate phylum Hydrothermae was recently proposed to accommodate the first genomic representatives of the *EM3* lineage (Jungbluth et al., 2017). This bacterium has

been observed in a high relative abundance in a thermophilic CSTR treating raw microalgae (Zamorano-López et al., 2019). These authors suggest that *EM3* is involved in microalgae cell-wall disruption, in substrate uptake after microalgae hydrolysis and also in beta-oxidation of intracellular lipids. This *Candidatus* Hydrothermae genus was observed for the first time in Sample #5 and was relatively abundant during all the periods (Figure 5.5a), indicating that it is an important bacterium for ACoD with primary sludge.

5.3.2.2. Archaea population

Within the archaeal domain, the Methanosarcinales order was dominant in the reactor during the four periods investigated and represented around 90% of the cumulative community relative abundance. *Methanoculleus and Methanospirillum* (Methanomicrobiales order), *uncultured Methanomassiliicoccaceae* (Methanomassiliicoccales order), and *Methanobrevibacter* (Methanobacteriales order) were observed in the thermophilic AnMBR (Figure 5.5b).

As shown in the bacterial analysis, the relative abundance of the abovementioned groups changed from the start-up period to PI, as seen in Figure 5.5b. In Sample #1, the total relative abundance was more divided among groups, while, from Sample #3, after the membrane installation, the Methanosarcinales order was the most predominant. The implementation of a membrane can offer a robust system with a stable microbial community through the complete retention of the biomass by the membrane, which is capable of degrading both microalgae and primary sludge while producing methane. One member related to Methanosarcinales (*Methanosaeta*) was the most predominant during all pseudo-steady states. Although *Methanosaeta* is commonly found in full-scale mesophilic and not in thermophilic digesters (Kirkegaard et al., 2017; Maus et al., 2016), it has already been isolated in thermophilic lab-scale reactors digesting microalgae (Greses et al., 2017; Zamorano-López et al., 2019). The presence of this dominant genus suggests acetoclastic methanogenesis as the main pathway for methane production after acetate consumption. This dominance is in accordance with the absence of VFA in the reactor's effluent during the pseudo-steady state (Figure 5.2a). However, hydrogenotrophic methanogenesis also contributed to methane production, although to a lesser extent, via Methanomicrobiales (*Methanoculleus* and *Methanospirillum*) and Methanobacteriales (*Methanobrevibacter*).

The strong presence of hydrogenotrophic *Methanoculleus* in Sample #14 (24.3% over archaea relative abundance) should be noted. An operational temperature problem occurred on Day 547, resulting in a VFA accumulation, as described in Section 5.3.1. Goberna et al. (2010) also found a high positive correlation between VFA concentration and *Methanoculleus*. The presence of this H_2 scavenger during this episode suggests its relation with syntrophic propionic or acetate-oxidising bacteria. Manzoor et al. (2016) described *Methanoculleus* as a syntrophic partner of mesophilic acetate-oxidising bacteria (SAOB). In the present work there was an increase (from 5% to 24%) in Sample #14 in the relative abundance of *W5* (Figure 5.5a), a syntrophic propionate oxidiser. As propionate degradation has been considered as a rate-limiting step in AD and propionate accumulation is a sign of process instability, the interaction between SPOB and hydrogenotrophic archaea would energetically facilitate the propionate degradation. The operating temperature peak forced a change in the microbial community and promoted certain links between microorganisms that recovered the system stability and reduced accumulated VFA. As can be seen in the last 3 samples of the PIV pseudo-steady state (Figure 5.5b), the microbial community changed from Sample #14 and *Methanosaeta* was again the dominant genus.

The microbial community was found to evolve from Sample #1. Since the system had been inoculated with mesophilic sludge, some thermophilic microorganisms such as *Candidatus* Hydrothermae genus, *Thermogutta* or *Fervidobacterium* appeared in the system after several weeks. A change in microbial diversity was also found from Sample #3, after membrane installation. The strong presence of hydrolytic, fermentative and proteolytic microorganisms is related to the significant content in protein of *Chlorella* microalgae and the presence of cellulose in both primary sludge and microalgae. The microbial community observed in the present work is similar to that in a thermophilic CSTR treating raw *Scenedesmus* (Greses, 2017), since microorganisms commonly found in microalgae digesters are present in both systems. However, due to the membrane, the AnMBR has greater taxonomic diversity and the different groups have different relative abundance. The remarkable contribution of a syntrophic network with hydrogen-producing bacteria such as *W5*, *Defluviitoga* or *Acetomicrobium* showed the importance of these groups in thermophilic microalgae and primary sludge co-digestion. The SPOB can act as partner of hydrogenotrophic methanogens, such as *Methanoculleus* or

Methanomassiliicoccaceae, which use H_2 and CO_2 to produce methane, but these SPOB also produce acetate, which can be used by the acetoclastic methanogen *Methanosaet*a, the dominant methanogen found during the pseudo-steady state in all the periods, to produce methane.

Overall, the AnMBR configuration in the thermophilic system allowed a good performance of the process, overcoming any VFA or TS accumulation caused by an increase in OLR and creating an adapted microbial community that was stable over time. Even some operational problems occurred, such as a feeding overloading during 7 days or a sudden raise in temperature of 15 ºC, the reactor showed no inhibition and it was self-recovered after around 20 and 40 days, respectively, demonstrating the robustness of the system. Co-digesting microalgae with primary sludge, joint to the incorporation of the membrane to the system resulted in a high microalgae degradation, which means that energy is being recovered from wastewater through methane production. Then, the thermophilic AnMBR configuration is a promising approach for industrial-scale application. However, due to the higher costs normally associated with the high temperature of operation (55 ºC) in thermophilic systems, an economic balance comparing mesophilic and thermophilic systems need to be considered for future implementation in real Waste Resource Recovery Facilities.

5.4. CONCLUSIONS

This study reveals for the first time the microbial community involved in a thermophilic microalgae co-digestion process and expands our understanding of the effect over microbial community of implementing a membrane to the system. The AnMBR configuration allowed working at high SRT (70 d), promoting a stable and robust microbial core capable of degrading the typically recalcitrant microalgae (*Chlorella* spp.) cell wall. The importance of syntrophic microorganisms such as *W5* or *Defluviitoga* for microalgae co-digestion was here highlighted. Primary sludge and microalgae biomass co-digestion produced 1.5 $L \cdot d^{-1}$ of methane-rich biogas, equivalent to a methane yield of 242 $mL CH_4 \cdot gCOD_{influent}^{-1}$. Although FAN inhibition was observed co-digesting microalgae in a CSTR, the AnMBR configuration enhanced the performance of the thermophilic ACoD, allowing the reactor operation at a higher OLR without FAN inhibition. The 3-fold OLR increase improved biodegradability by 35%. The stability

reached over time (700 d) in terms of biological process and microbial community in a semi-continuous thermophilic system is key for proposing a future full-scale application.

Contributions

RSG: Carried out the experiments, analysed and interpreted data, draft and wrote the manuscript. LB: planned and designed the experiments, analysed microbial data, revised the paper, obtained funding. AB: planned and designed experiments, analysed biological data, revised the article. AS: planned and designed the experiments, revised the article, obtained funding.

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Statement of informed consent human/animal rights

No conflicts, informed consent, or human or animal rights are applicable to this study

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Supplementary information Chapter V

Table 5.S1: Mean ± standard deviation (n=3) of the predominant bacteria and archaeal domain in the four periods investigated in the thermophilic AnMBR plus the composition of start-up period samples determined by 16S rRNA gene sequencing.

	Start-up		**PI**			**PII**	**PIII**	**PIV**	
Taxonomy assignment	**#1**	**#2**	**#3**	**#4**	**#5 #6 #7**	**#8 #9 #10**	**#11 #12 #13**	**#14**	**#15 #16 #17**
d.-Bacteria; p.-Coprothermobacteraeota; c.-Coprothermobacteria; o.-Coprothermobacterales; f.-Coprothermobacteraceae; g.-*Coprothermobacter*	5.6	22.3	24.8	30.2	23.7±6.8	17.4±0.7	21.6±4.9	14.4	14.3±3.1
d.-Bacteria; p.-Thermotogae; c.-Thermotogae; o.-Thermotogales; f.-Fervidobacteriaceae; g.-*Fervidobacterium*	n.d.	n.d.	4.4	14.3	11.6±3.7	19.2±0.4	19.6±4.9	15.7	22.1±4
d.-Bacteria; p.-Hydrothermae c.- Hydrothermae_cl; o.- Hydrothermae_or; f.- Hydrothermae_fa; g.- *Hydrothermae_ge*	n.d.	n.d.	n.d.	n.d.	16.7±3.1	16.4±0.8	3.4±0.3	1.4	10.6±2.5
d.-Bacteria; p.-Thermotogae; c.-Thermotogae; o.-Petrotogales; f.-Petrotogaceae; g.-*Defluviitoga*	n.d.	n.d.	n.d.	3.5	5.4±0.7	5.7±0.6	7.6±2.4	2.0	3.4±0.7
d.-Bacteria; p.-Cloaciomonetes; c.-Cloacimonadia; o.-Cloacimonadales; f.-Cloacimonadaceae; g.-*W5*	0.0	0.0	0.0	0.0	0.0	0.0	3.3±2.9	23.8	4.9±2.5
d.-Bacteria; p.-Synergistetes; c.-Synergistia; o.-Synergistales; f.-Synergistaceae; g.-*Acetomicrobium*	1.0	2.0	2.5	2.5	2.2±0.1	3.5±0.4	3.2±1.4	3.0	3.0±0.1
d.-Bacteria; p.-Epsilonbacteraeota; c.-Campylobacteria; o.-Campylobacterales; f.-Arcobacteraceae; g.-*Arcobacter*	19.7	8.9	1.8	1.3	1.7±1.6	0.8±0.7	n.d.	n.d.	n.d.
d.-Bacteria; p.-Atribacteria; c.-Caldatribacteriia; o.-Caldatibacteriales; f.-Caldatribateriaceae; g.-Candidatus_Caldatribacterium	n.d.	n.d.	n.d.	2.0	2.0±0.3	2.2±0.4	2.0±0.3	1.2	1.7±0.3

(Continues in next page)

	Start-up		PI			PII	PIII	PIV	
Taxonomy assignment	**#1**	**#2**	**#3**	**#4**	**#5 #6 #7**	**#8 #9 #10**	**#11 #12 #13**	**#14**	**#15 #16 #17**
d.-Bacteria; p.-Planctomycetes; c.-Planctomycetacia; o.-Pirellulales; f.-Pirellulaceae; g.-*Thermogutta*	n.d.	n.d.	n.d.	4.0	1.1±1.1	2.4±0.9	1.8±0.8	n.d.	1.7±0.4
d.-Bacteria; p.-Caldiserica; c.-Caldisericia; o.-Caldisericales; f.-Caldisericaceae; g.-*Caldisericum*	n.d.	n.d.	n.d.	n.d.	2.0±2.0	1.2±0.0	1.5±1.3	n.d.	2.1±0.3
d.-Bacteria; p.-Proteobaceria; c.-Gammaproteobacteria; o.-Betaproteobacteriales; f.-Hydrogenophlaceae; g.-*Tepidiphilus*	n.d.	n.d.	14.0	2.5	n.d.	n.d.	n.d.	n.d.	n.d.
d.-Bacteria; p.-Bacteroidetes; c.-Bacteroidia; o.-Bacteroidales; f.-Bacteroidaceae; g.-*Proteiniphilum*	n.d.	n.d.	10.8	3.5	n.d.	n.d.	n.d.	n.d.	n.d.
d.-Bacteria; p.-Bacteroidetes; c.-Bacteroidia; o.-Bacteroidales; f.- Dysgonomonadaceae; g.-*Bacteroides*	4.9	5.0	1.3	n.d.	0.6±0.1	n.d.	n.d.	n.d.	n.d.
d.-Bacteria; p.-Proteobaceria; c.-Gammaproteobacteria; o.-Pseudomonadales; f.-Pseudomonadaceae; g.-*Pseudomonas*	5.5	4.4	1.3	1.8	n.d.	n.d.	n.d.	n.d.	n.d.
d.- Archaea; p.-Euryarchaeota; c.-Methanomicrobia; o.-Methanosarcinales; f.-Methanosaetaceae *Methanosaeta*	0.0	0.0	0.0	0.0	1.2±0.3	1.5±0.2	0.5±0.1	0.2	1.1±0.4
d.- Archaea; p.-Euryarchaeota; c.-Methanomicrobia; o.-Methanomicrobiales; f.-Methanomicrobiaceae; g. *Methanoculleus*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0
d.- Archaea; p.-Euryarchaeota; c.-Methanomicrobia; o.-Methanosarcinales; f.-Methanosarcinaceae; g. *Methanosarcina*	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0

Non-detected OTU (n.d.). The taxonomic level for each $OTU_{0.97}$ is indicated as follows: phylum (p.), class (c.), order (o.), family (f.) and genus (g.)

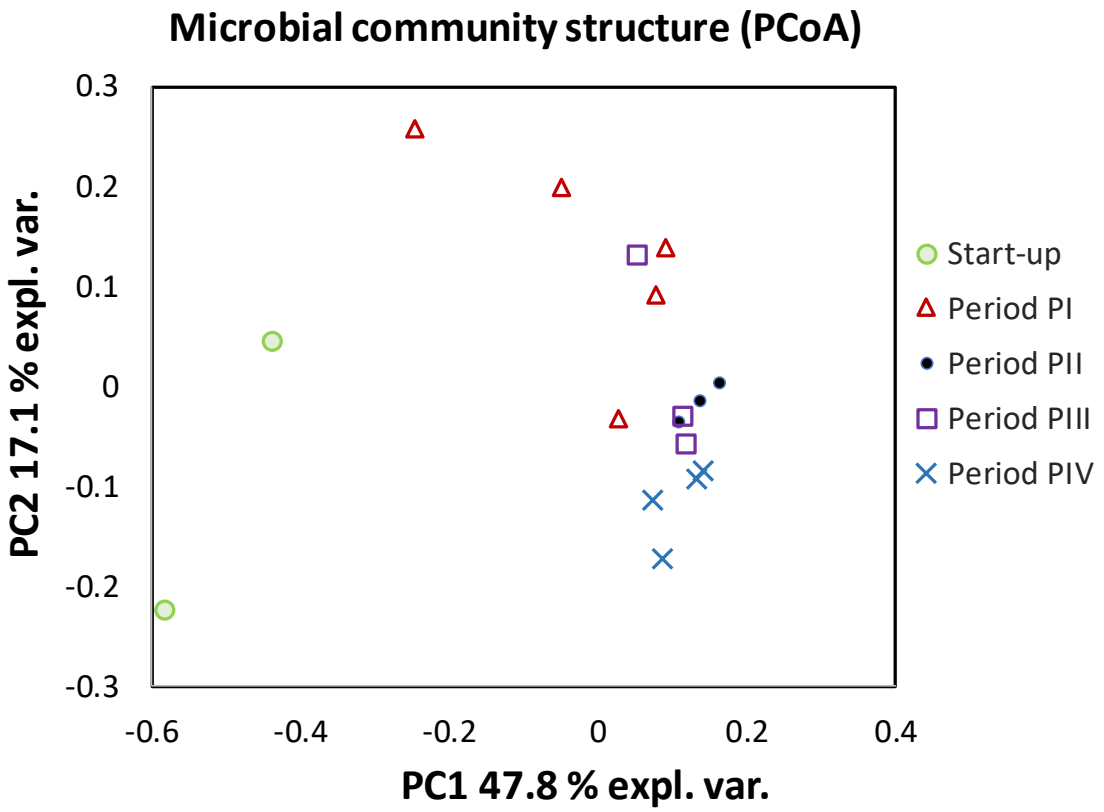

Figure 5.S1: Principal coordinate analysis (PCoA) based on the Bray-Curtis dissimilarity between samples for each period showing the microbial structure shifted after start-up and membrane installation. Explained variance of each component is shown in percentage in each axis.

Anaerobic membrane bioreactor (AnMBR) scale-up from laboratory to pilot-scale for microalgae and primary sludge co-digestion: biological and filtration assessment

6. ANAEROBIC MEMBRANE BIOREACTOR (AnMBR) SCALE-UP FROM LABORATORY TO PILOT-SCALE FOR MICROALGAE AND PRIMARY SLUDGE CO-DIGESTION: BIOLOGICAL AND FILTRATION ASSESSMENT

Abstract: This research work proposes the scale-up evaluation in terms of biological and filtration performance from laboratory to pilot-scale of an anaerobic membrane bioreactor (AnMBR) co-digesting raw microalgae and primary sludge. Best operating conditions for this scale-up were energetically and economically assessed based on laboratory results. Economic balance showed 3% higher annual costs when operating a reactor at 100 d solids retention time (SRT) compared to 70 d SRT. Energetic balance showed a 5.5-fold increase in heat demand working at thermophilic temperature comparing to mesophilic. The AnMBR operating conditions were set at 70 d SRT and 35 ºC. The pilot-scale and lab-scale co-digesters performed similarly in terms of biogas production and system stability. 154 $mL_{biogas} \cdot d^{-1} \cdot L^{-1}_{reactor}$ were produced at pilot-scale, corresponding to methane yield of 215 $mL_{CH_4} \cdot gCOD_{inf}^{-1}$. AnMBR filtration at both laboratory and pilot-scale showed stability working at permeate fluxes of 4.2-5.8 $L \cdot m^{-2} \cdot h^{-1}$.

Keywords: Anaerobic membrane bioreactor (AnMBR); anaerobic co-digestion (ACoD); microalgae; membrane; methane.

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6.1. INTRODUCTION

The traditional scheme for wastewater treatment is currently being replaced by more efficient, competitive and less resource-demanding technologies. In recent years, a transition has been observed from classical Waste Water Treatment Plants (WWTPs) to novel Water Resource Recovery Facilities (WRRFs) where sewage is not only treated as a waste but as a source for raw valuable resources, giving rise to environmental and economic benefits (Donoso-Bravo et al., 2020; Seco et al., 2018).

Anaerobic digestion (AD) is part of this new WRRF concept since no oxygen is needed for organics removal, fewer biosolids are produced and biodegradable compounds are converted into the energy carrier methane. Specifically, anaerobic membrane bioreactors (AnMBRs) are being considered a feasible alternative to conventional treatments since they can combine AD with membrane filtration, offering several advantages: (i) decoupling of hydraulic retention time (HRT) from solids retention time (SRT), which avoids microorganisms from being washout due to biomass retention in the membrane; (ii) high efficiency in terms of organic matter and suspended solids removal, offering a high-quality effluent in which microbiological contamination has also been eliminated (bacteria, microalgae, parasites) (Robles et al., 2018); (iii) resource recovery potential; and (iv) lower energy costs.

However, when operating a membrane-based system some issues should be considered. The principal concern is membrane fouling, generated by cell's secretions and compounds that accumulate in the membrane surface, creating a cake-layer, which decreases permeate flux due to a reduction in membrane permeability thereby rising energy consumption (Gong et al., 2019). Fouling is especially troubling when filtrating substrates with high suspended solids and organic matter such as microalgae biomass (Wang et al., 2019). The accumulation of soluble microbial products (SMPs) inside the reactor is reported to be key in membrane fouling since these molecules could lead to membrane pore blocking (Aslam et al., 2018). Physical cleaning could be a solution for cake layer removal but frequently a chemical cleaning is needed, shortening membrane lifetime. However, irrecoverable fouling could occur, which is not removed by chemical or physical cleaning. Robles et al. (2013) reported that gas sparging intensity and backwash frequency are the physical variables that affect membrane cleaning most when

treating urban wastewater. Indeed, when membranes were operated at sub-critical fluxes and a suitable physical cleaning schedule was performed, no meaningful irreversible or irrecoverable fouling were detected, thus avoiding chemical cleaning (Robles et al., 2013). Lei et al. (2018) found that the combination of gas sparging to the membranes with backwashing could sufficiently control internal fouling, although pore blocking partially led to irreversible fouling.

The AnMBR effluent presents a low content in solids but still presents high nutrient concentrations, which can be recovered by microalgae cultivation. These microalgae could be later anaerobically digested, giving rise to a digested sludge enriched in nutrients that could be recovered, completing a scheme in which energy and nutrients are regained from sewage. However, microalgae AD still presents some hindrances, such as the high resistance to biodegradation that their cell walls present or the low microalgae C/N ratio (due to the high nitrogen content) that it is not the optimum one for AD process (Giménez et al., 2018). These drawbacks could be addressed by adding a carbon-rich co-substrate such as sludge, since this kind of substrate could raise C/N ratio, avoiding a possible inhibition by ammonia and therefore increasing process stability and AD performance (Wang et al., 2013; Yen and Brune, 2007). There are several researches on anaerobic co-digestion (ACoD) of microalgae with various types of sludge (Mahdy et al., 2015; Olsson et al., 2014; Wang et al 2013), but are based on microalgae cultivated using synthetic media or filtered sewage, which results could be not reliable when operating industrial-scale anaerobic digesters using raw wastewater. Nevertheless, recent studies have evaluated the ACoD of wastewater-grown microalgae and primary sludge at lab-scale, observing promising results: Solé-Bundó et al. (2019) observed how methane production was enhanced by 65% when co-digesting microalgae and primary sludge (in a proportion 25/75% based on total volatile solids (TVS)), compared to microalgae mono-digestion, working in a lab-scale CSTR at mesophilic conditions. Serna-García et al. (2020a and 2020b) studied the anaerobic co-digestion of microalgae and primary sludge (in a proportion 38/62% based on TVS) in a lab-scale AnMBR working at mesophilic and thermophilic conditions, observing high biodegradability percentages (73 and 69%, respectively).

Previous studies (Serna-García et al. 2020a and 2020b) on microalgae and primary sludge ACoD at laboratory-scale showed promising results. Nevertheless, to the best knowledge

of the authors, no investigation on microalgae ACoD has been carried out at pilot-scale and none of the laboratory studies have assessed the filtration process performance. The aim of this work was to scale-up an AnMBR treating wastewater-grown harvested microalgae and primary sludge from laboratory to pilot-scale to evaluate the feasibility of this technology for future industrial applications. Biological and filtration process were first evaluated at lab-scale and best operating conditions were chosen to scale-up the laboratory layout outdoor at pilot-scale. The potential recovery of valuable resources (energy and nutrients) was evaluated.

6.2. METHODS

6.2.1. Substrates

ACoD of microalgae and primary sludge was carried out both at laboratory and pilot-scale. Primary thickened sludge coming from the Cuenca del Carraixet full-scale WWTP (Alboraya, Valencia, Spain) was used as feeding co-substrate. It was sieved through a 0.5 mm mesh size sieve to discard large particles and diluted to achieve the desired chemical oxygen demand (COD) concentration. Microalgae biomass harvested from a membrane photobioreactor pilot plant, which was fed with the effluent of an AnMBR treating urban wastewater (see González-Camejo et al. (2017) for the whole description), was also used as a co-substrate. Microalgae were concentrated in an external cross-flow filtration tubular module containing ultrafiltration membrane fibres (HF 5.0-43-PM500, ROMICON® Koch Membrane Systems, USA), with a pore size of 500 kDa molecular weight cut-off and a filtration area of 2.1 m^2. The final composition of the mixture fed to the AnMBR was the following: 38 and 62% of TVS for harvested microalgae and primary sludge, respectively, according to findings from a previous study (Seco et al., 2018).

6.2.2. Experimental set-up and operation

6.2.2.1. Lab-scale operation

An AnMBR with a total volume of 14 L (up to 9 L working volume) was used for ACoD trials at laboratory-scale (Figure 6.1a) (see Serna-García et al. (2020a) for the complete description). The reactor was connected to an external hollow-fibre ultrafiltration membrane (UHF), (0.03 μm pore size, PURON® Koch Membrane Systems, USA), with a filtration area of 0.44 m^2. The system was operated during 390 days at mesophilic

conditions (35 ºC). The operating SRT and HRT were set at 70 and 30 d, respectively, with an organic loading rate (OLR) of 0.5 gCOD·L^{-1}·d^{-1}. It has to be highlighted that OLR was temporarily incremented to 1.11 gCOD·L^{-1}·d^{-1} from Day 182 to 189, due to a substrate increased concentration. The reactor was inoculated with a mesophilic sludge from a lab-scale reactor co-digesting microalgae and primary sludge. An MGC milligas counter (Ritter, Germany) was used to measure the biogas production and gas volumes were normalised to conditions of 1 atm and 0 ºC of pressure and temperature, respectively.

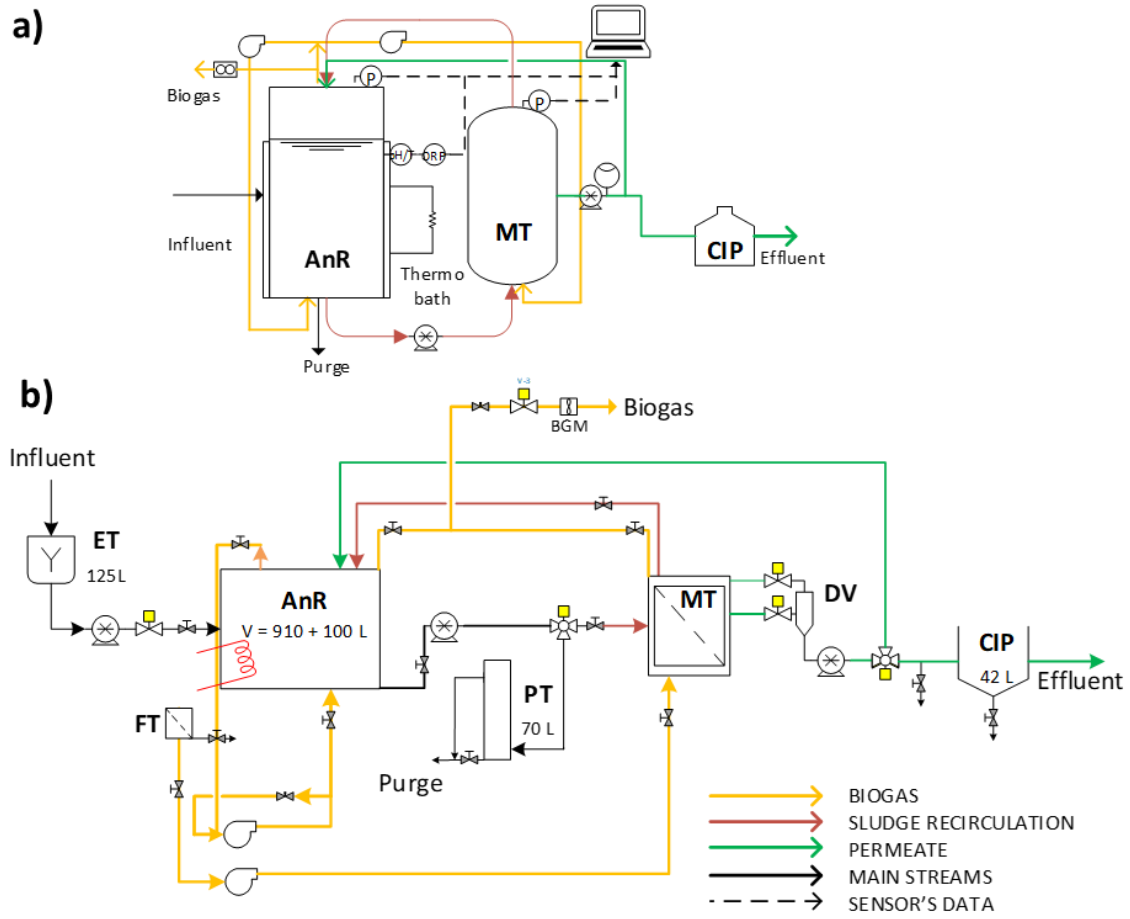

Figure 6.1. Lab-scale anaerobic membrane bioreactor scheme (a), anaerobic co-digestion pilot plant scheme (b). ET: equalisation tank, AnR: anaerobic reactor, FT: foam tank, PT: purge tank, MT: membrane tank, DV: degasification vessel, CIP: clean-in-place tank, BGM: biogas meter.

6.2.2.2. Pilot-scale operation

For pilot-scale ACoD trials, the experimental set-up was very similar to the one used at lab-scale (Figure 6.1b). The co-digestion pilot plant consisted of a reactor with a total

volume of 1010 L (up to 910 L working volume) coupled to a 1 L membrane tank (MT) fitted with a 0.44-m^2 UHF unit (0.03 µm pore size, PURON® KMS, USA). The different co-substrates were mixed in a 125 L equalisation tank before being fed to the system. A 42 L clean-in-place (CIP) tank, a 70 L purge tank (PT) and a 70 L foam trap (FT) were also included as main elements of the plant. An instrumentation, control and automation (ICA) system was implemented to assure the correct pilot plant operation. To control the digester's temperature, three external resistances were installed. Total biogas produced was measured by a biogas meter (BK-G4M, Elster, USA) and gas volumes were normalised to conditions of 1 atm and 0 ºC of pressure and temperature, respectively. A fraction of the generated biogas was recycled from the top of the reactor to the bottom, employing a blower, to assure the correct mixing of the sludge and to facilitate the stripping of the gases from the liquid phase. Another fraction of the biogas produced was recycled through another blower from the top of the reactor to the bottom of the UHF, minimising the biofilm formation. Sludge was continuously recycled through the MT where the effluent was obtained by vacuum filtration, after passing through a degasification vessel (DV).

The pilot plant was run for a period of 360 days and operating conditions were kept equal to the ones used at laboratory scale: 35 ºC, 70 d of SRT, 30 d of HRT and 0.5 g $COD \cdot L^{-1} \cdot d^{-1}$ of OLR. The AnMBR was inoculated with anaerobic sludge coming from a full-scale mesophilic digester.

6.2.3. Membrane operation

Membrane operation was similar in both laboratory and pilot-scale reactors, programmed to operate in three stages: filtration, relaxation and backwash. Gas scouring was employed to control and minimize membrane fouling. The permeate flux (J) and achieved trans-membrane pressure (TMP) were on-line monitored using a multichannel data acquisition picoLog 1000 series (Pico Technology, UK).

6.2.3.1. Lab-scale operation

Lab-scale filtration was carried out discontinuously (permeate was obtained once a day in order to keep the desired HRT) during the first 360 days of AnMBR operation. To evaluate AnMBR scale-up, filtration experiments were carried out during the last 30 days of AnMBR operation at a 20 ºC-normalised permeate flux (J_{20}) of 5.8 $L \cdot m^{-2} \cdot h^{-1}$ (LMH)

and a specific gas demand (SGD) of 0.15 $N \cdot m^3 \cdot m^{-2} \cdot h^{-1}$. Baerdemaeker et al. (2013) compared filtration-relaxation (F-R) with filtration-backwash (F-B) performance in microalgae harvesting, obtaining stable filtration behaviour and no decline in permeability in the F-B experiment. Based on this previous experience, the relaxation period was removed for laboratory trials, conducting a backwash every cycle. The filtration and backwash times were 90 and 40 s, respectively.

With the aim of determining possible increases in the irreversible fouling, the filtration TMP and permeability were evaluated periodically at three different fluxes (3.5, 7.0 and 10.5 LMH). The evaluation consisted in setting the J_{20} during three filtration cycles and determining the maximum TMP (TMP_{MAX}) and permeability under those conditions. Thus, a periodic raise on the achieved TMP_{MAX} or a decrease in the permeability would denote a growth in the membrane irreversible fouling, being more critical at the highest fluxes employed. This procedure was performed twice a week coinciding with the SMPs and viscosity determination (see Section 6.2.4 for further information).

6.2.3.2. Pilot-scale operation

Filtration process at pilot-scale was evaluated during the whole experimental period and filtration experiments were carried out during the last days of AnMBR operation, once the pseudo-steady state was achieved and maintained (from Day 260), to know the incidence of different parameters over filtration process. The membrane operation was automated to enable the study of various backwash and relaxation frequencies and durations. A basic F-R cycle was established and taken as a basis to schedule the frequency of the remaining stages. Two additional stages were also considered to remove the accumulation of biogas at the top of the fibres (degasification) and inside the DV (ventilation). During the first 260 days of ACoD pilot plant operation, filtration was carried out at an average J_{20} of 3.5 LMH and a filtration time of 180 s. During the last 100 days of operation, the pilot-scale filtration was performed under four different conditions (Experiments I to IV, Table 6.1). A membrane physical cleaning (consisting of pressure washing) was carried out after every experiment. In these four experiments, relaxation and backwash times were 30 s and 45 s, respectively. A backwash was conducted every F-R cycle in Experiments I and II, but every two cycles in Experiments III and IV.

Table 6.1. Experimental conditions for the four filtration experiments carried out at pilot-scale.

	Specific gas demand ($N \cdot m^3 \cdot m^{-2} \cdot h^{-1}$)	**Permeate flux** ($L \cdot m^{-2} \cdot h^{-1}$)	**Recirculation ratio**	**Filtration time** (s)
Experiment I	0.6244 ± 0.0005	5.6 ± 0.3	1.81 ± 0.00	300
Experiment II	0.151 ± 0.002	5.5 ±0 .7	1.7 ± 0.2	160
Experiment III	0.15 ± 0.02	4.18 ± 0.04	1.92 ± 0.05	160
Experiment IV	0.32 ± 0.01	4.22 ± 0.03	1.81 ± 0.04	160

6.2.4. Analytical methods

Samples were collected thrice a week from the reactor and influent streams to evaluate the biological process performance. Total COD (COD), Total Solids (TS), TVS, Total Suspended Solids (TSS), Volatile Suspended Solids (VSS), Total Nitrogen (TN), Ammonium (NH_4-N), Nitrate (NO_3-N), Nitrite (NO_2-N), Total Phosphorus (TP), Phosphate (PO_4-P), Sulphide (S^{2-}) and Sulphate (SO_4-S) were determined in triplicate according to Standard Methods (APHA *et al*., 2012). Volatile Fatty Acids (VFA) and Alkalinity (Alk) were measured in triplicate by titration in accordance with Moosbrugger et al. (1993).

Microalgae and primary sludge composition (C, N, H and S) was analysed by energy-dispersive X-ray spectroscopy (EDS) in an XL-30 ESEM (Philips, Netherlands). Substrate samples were attached to the Scanning Electron Microscopy (SEM) stub using silver lacquer and then introduced into the XL-30 ESEM. Pressure was reduced to 10^{-5} bar, after which the sample surface was visualised and an area was selected for microanalysis.

The methane percentage in the biogas was determined thrice a week using a gas chromatograph equipped with a Flame Ionisation Detector (GC-FID, Agilent Technologies, USA). 1 mL of biogas was collected from the top of the reactor by a gas-tight syringe and injected into a 15 m × 0.53 mm × 1 μm TRACER column (Teknokroma,

Spain) which was operated at 40 °C. Helium was the carrier gas at a flow-rate of 40 $mL \cdot min^{-1}$. Pure methane (99.99%) was used as a standard.

To evaluate the filtration performance in the lab-scale filtration membrane, the viscosity and SMPs content in mixed liquor were evaluated twice a week. The amount of SMPs was attributed only to the protein and carbohydrate concentrations. For protein determination, a commercial Total Protein Kit, Micro Lowry, Peterson's Modification (Sigma-Aldrich, USA) was employed based on the Lowry-Peterson method (Peterson, 1977). For the carbohydrate determination, the Dubois method (Dubois et al., 1956) was used. Viscosity was determined by a Cannon-Fenske viscometer (Series 200, Merck, Germany).

6.2.5. Process performance indicators

6.2.5.1. Anaerobic process efficiency

The biological process efficiency was evaluated using equations reported previously in Serna-García et al. (2020a) regarding biodegradability percentage and methane production yields.

6.2.5.2. Energy and economic balance

An energy and economic balance, according to the model proposed by Seco et al. (2018), was performed in order to select the best operating conditions for scaling-up the process. For energy balance, only heating requirements were considered. The heating requirements (Q_{total}) were calculated considering the energy necessary for increasing the influent temperature and the reactor's heat losses (Eq. 6.1)

$$Q_{total} = c_P \cdot q \cdot \rho_{sludge} \cdot \left(T_{reactor} - T_{inflow}\right) + U \cdot S \cdot (T_{reactor} - T_{outside}) \left(\frac{kcal}{h}\right)$$

(Eq. 6.1)

c_P ($kcal \cdot kg^{-1} \cdot K^{-1}$) being the specific heat; q ($m^3 \cdot h^{-1}$) the input flow rate; ρ_{sludge} ($kg \cdot m^{-3}$) the sludge density; ($T_{reactor}$-T_{inflow}) (K) the temperature difference between the inflow and the reactor; U ($kcal \cdot h^{-1} \cdot m^{-2} \cdot K^{-1}$) the overall heat transfer coefficient obtained by Eq. 6.2; S (m^2) the reactor surface, and ($T_{reactor}$-$T_{outside}$) the temperature difference between the reactor and the outside.

$$U = \frac{1}{\frac{\delta_{reactor}}{K_{reactor}} + \frac{1}{h_{air}}} \left(\frac{kcal}{h \cdot m^2 \cdot K}\right) \quad \text{(Eq. 6.2)}$$

$\delta_{reactor}$ (m) being the reactor thickness; $K_{reactor}$ (kcal·h^{-1}·m^{-1}·K^{-1}) the reactor material conductivity, and h_{air} (kcal·h^{-1}·m^{-1}·K^{-1}) the air convective heat transfer coefficient.

Heat recovery was calculated as the heat recovered from methane considering a combined heat and power (CHP) unit (Q_{CHP}; Eq. 6.3). Since the amount of dissolved methane in the effluent was low and no treatment was designed for its recovery, it was not considered in this calculation.

$$Q_{CHP} = \frac{V_{BG} \cdot (\%CH_4 \cdot CV_{CH_4}) \cdot \%q_{eff\ CHP}}{1000 \cdot 24 \cdot 4.187} \cdot \%q_{exch} \left(\frac{Kcal}{h}\right) \quad \text{(Eq. 6.3)}$$

V_{BG} (L·d^{-1}) being the biogas flow rate; $\%CH_4$ the methane richness (%); CV_{CH4} (kJ·m^{-3}) the methane calorific power; $\%q_{eff}$ CHP the heat efficiency of the CHP system (%), and $\%q_{exch}$ the heat exchanger efficiency (%). A q_{eff} CHP of 45% and a q_{exch} of 65% were considered according to Seco et al. (2018).

Total energy demanded for the process (Q_{demand}) was calculated as the difference between total heat requirements and heat recovered from CHP (Eq. 6.4).

$$Q_{demand} = (Q_{TOT} - Q_{CHP}) \cdot 0.0011622 \left(\frac{kWh}{m^3}\right) \quad \text{(Eq. 6.4)}$$

For economic balance, calculations were related to construction costs (C_C), equipment costs (pumps and blowers), land cost and annual depreciation costs (D_C), according to Eq. 6.5 and Eq. 6.6, respectively.

$$C_C = Concrete\ wall\ price \cdot V_R + Concrete\ slab\ price \cdot V_C\ (k€) \quad \text{(Eq. 6.5)}$$

concrete wall price and concrete slab price (k€·m^{-3}) being the prices of the materials needed for the reactor construction, V_R (m^3) the annular volume of the reactor, and V_C (m^3) the volume of the reactor cover.

$$D_C = \frac{C_C + Equipment\ cost + Land\ cost}{t}\ (k€) \quad \text{(Eq. 6.6)}$$

t (years) being the depreciation period. In this work, a depreciation period of 20 years was chosen for calculations.

For both economic and energy balance, some considerations relating to reactor characteristics and construction materials were assumed for the operation scales. Electricity production from biogas generated (W_{BG}) was also considered (Eq. 6.7).

$$W_{BG} = \frac{V_{BG} \cdot \%\, CH_4 \cdot CV_{CH4} \cdot \eta_{CHP}}{1000 \cdot 24 \cdot 3600}\ (kW) \quad \text{(Eq. 6.7)}$$

η_{CHP} being the methane electricity conversion efficiency by the CHP system. A η_{CHP} of 0.35 was considered according to Seco et al. (2018).

6.2.5.3. Membrane performance

The feasibility of the filtration process was evaluated according to the 20 ºC-normalised membrane permeability (K_{20}). A simple filtration model was employed to calculate the K_{20} values (Eq. 6.8). Since permeate viscosity strongly affects the filtration performance, a temperature correction factor (f_T) was included in the model (Eq. 6.9) which can be also used to determine the 20 ºC-normalised permeate flux (J_{20}), (Eq. 6.10).

$$K_{20} = \frac{J_T \cdot f_T}{TMP}\ (LMH \cdot bar^{-1}) \quad \text{(Eq.6. 8)}$$

$$f_T = e^{-0.0239\,(T-20)} \quad \text{(Eq. 6.9)}$$

$$J_{20} = J \cdot f_T\ (LMH) \quad \text{(Eq. 6.10)}$$

6.3. RESULTS AND DISCUSSION

6.3.1. Laboratory-scale

Microalgae and primary sludge, substrates in the co-digestion system were characterised (Table 6.2). Microalgae showed a low C/N ratio (5.2), due to the high nitrogen content, and primary sludge showed a higher C/N ratio (9.5), but it was not in the recommended range (20-25) for an AD process (Yen and Brune, 2007). The main parameters extracted from the AnMBR performance are summarized in Table 6.3. The AnMBR was operated during 390 days at lab-scale and pseudo-steady state was achieved after 220 days of operation (Figure 6.2). Alkalinity, VFA and pH in the digester showed normal values for a well-stabilised digester (Table 6.3). Even when the OLR was temporarily incremented to 1.11 $gCOD \cdot L^{-1} \cdot d^{-1}$ from Day 182 to 189, pH was around 7.1 and no accumulation of VFA or TS was observed (Figure 6.2c). The low VFA concentration is an indicator of the

good balance between acidogenesis, acetogenesis and methanogenesis processes. Indeed, previous studies carried out in the same reactor (Serna-García et al., 2020a) found a stable microbial community capable of producing methane at a high rate. The volumetric production of biogas was 1.62 $L \cdot d^{-1}$, with a relatively constant biogas percentage of 70% (Figure 6.2a) resulting in a methane yield of 225 $mLCH_4 \cdot gCOD_{inf}^{-1}$ (391 $mLCH_4 \cdot gTVS_{inf}^{-1}$). Around 65% of the COD was degraded into biogas. Then, even the C/N ratio obtained was not high (Table 6.2), the AnMBR co-digestion system showed a good performance since primary sludge is a more easily biodegradable substrate, and mixing it with microalgae seems to have positive synergism effects, enhancing methane production compared to microalgae or primary sludge mono-digestion (Serna-García et al., 2020a).

Table 6.2. Microalgae and primary sludge characterisation at lab-scale.

Parameter	Unit	Microalgae	Sludge
C	%	47.5 ± 0.3	42.0 ± 1.2
N	%	9.2 ± 0.1	4.4 ± 0.7
S	%	0.45 ± 0.18	1.1 ± 0.5
H	%	7.1 ± 0.4	6.8 ± 0.7
COD	$mgCOD \cdot L^{-1}$	9739 ± 187	22430 ± 1190
TSS	$mgTSS \cdot L^{-1}$	6240 ± 340	14570 ± 620
VFA	$mgCH_3COOH \cdot L^{-1}$	102 ± 66	1060 ± 350
Alk	$mgCaCO_3 \cdot L^{-1}$	360 ± 120	229 ± 140
T-N	$mgN \cdot L^{-1}$	478 ± 53	478 ± 45
NH_4-N	$mgN \cdot L^{-1}$	28 ± 16	100 ± 26
T-P	$mgP \cdot L^{-1}$	103 ± 54	156 ± 52
PO_4-P	$mgP \cdot L^{-1}$	15 ± 10	68 ± 17
SO_4-S	$mgS \cdot L^{-1}$	101 ± 22	42 ± 18

C: carbon; N: nitrogen; S: sulphur; H: hydrogen; COD: chemical oxygen demand; TSS: total suspended solids; VFA: volatile fatty acids; Alk: alkalinity; TN: total nitrogen; NH_4-N: ammonium; TP: total phosphorus; PO_4-P: phosphate; SO_4-S: sulphate

Table 6.3. Main anaerobic co-digestion performance data including mean and standard deviation (n=3) values retrieved from the pseudo-steady state conditions reached at laboratory and pilot-scale and comparison with previous studies.

	Serna-García et al., 2020a	**Serna-García et al., 2020b**	**Present work**	**Present work**
Operating conditions				
Scale	Lab-scale	Lab-scale	Lab-scale	Pilot-scale
Temperature (°C)	35.4 ± 0.6	54.6 ± 0.7	35.4 ± 0.5	35.2 ± 0.5
SRT (d)	99.7 ± 0.5	70.4 ± 0.8	70.2 ± 0.6	70.2 ± 4
OLR ($gCOD \cdot L^{-1} \cdot d^{-1}$)	0.50 ± 0.01	0.50 ± 0.03	0.52 ± 0.02	0.58 ± 0.12
Biogas production and removal efficiencies				
Biodegradability (%)	73.1 ± 2.6	69.2 ± 2.0	64.6 ± 3.1	61.7 ± 2.0
TVS removal (%)	70.0 ± 3.3	69.2 ± 4.9	60.8 ± 3.4	63.6 ± 2.4
Methane yield ($mLCH_4 \cdot gCOD_{inf}^{-1}$)	256 ± 10	242 ± 8	225 ± 10	215 ± 6
Methane biogas content (%)	68.4 ± 2.5	70.5 ± 1.8	70.2 ± 2.4	69.1 ± 1.2
Influent				
Microalgae Feedstock	*Chlorella, Scenedesmus*	*Chlorella*	*Chlorella*	*Chlorella, Scenedesmus*
TN ($mg \cdot L^{-1}$)	420 ± 140	n.a.	478 ± 47	578 ± 113
NH_4-N ($mg \cdot L^{-1}$)	80 ± 27	62.1 ± 23.7	58 ± 19	97 ± 20
TP ($mg \cdot L^{-1}$)	140 ± 19	n.a.	126 ± 40	137 ± 17
PO_4-P ($mg \cdot L^{-1}$)	42.5 ± 9.4	17.3 ± 6.1	36.3 ± 14.1	48.0 ± 14.1
SO_4-S ($mg \cdot L^{-1}$)	48.6 ± 28.8	48.5 ± 4.7	75.2 ± 17.3	194 ± 63
TS ($mg \cdot L^{-1}$)	11600 ± 140	12000 ± 400	11700 ± 180	13100 ± 2000
TSS ($mg \cdot L^{-1}$)	1000 ± 400	9400 ± 300	10039 ± 536	11558 ± 1323
COD ($mg \cdot L^{-1}$)	15600 ± 200	14900 ± 700	15014 ± 541	15895 ± 1682
Effluent				
pH	6.9 ± 0.3	n.a.	7.1 ± 0.3	7.2 ± 0.1
VFA ($mgCH_3COOH \cdot L^{-1}$)	<D.L.	<D.L.	<D.L.	13.1 ± 15.4
Alk ($mgCaCO_3 \cdot L^{-1}$)	2030 ± 120	2030 ± 110	2070 ± 200	2060 ± 110
TS ($mg \cdot L^{-1}$)	13000 ± 350	9500 ± 180	12300 ± 540	11450 ± 660
TN ($mg \cdot L^{-1}$)	857 ± 176	648 ± 164	585 ± 81	685 ± 80
NH_4-N ($mg \cdot L^{-1}$)	401 ± 42	426 ± 42	423 ± 37	397 ± 33
TP ($mg \cdot L^{-1}$)	148 ± 19	81 ± 12	107 ± 28	145 ± 16
PO_4-P ($mg \cdot L^{-1}$)	28.6 ± 2.2	20.8 ± 0.7	24.7 ± 3.7	36.6 ± 6.1

OLR: organic loading rate; TVS: total volatile solids; CH_4: methane; TN: total nitrogen; NH_4-N: ammonium; TP: total phosphorus; PO_4-P: phosphate; SO_4-S: sulphate; TS: total solids; TSS: total suspended solids; VFA: Volatile fatty acids; Alk: alkalinity; D.L.: Detection Limit; n.a.: not available.

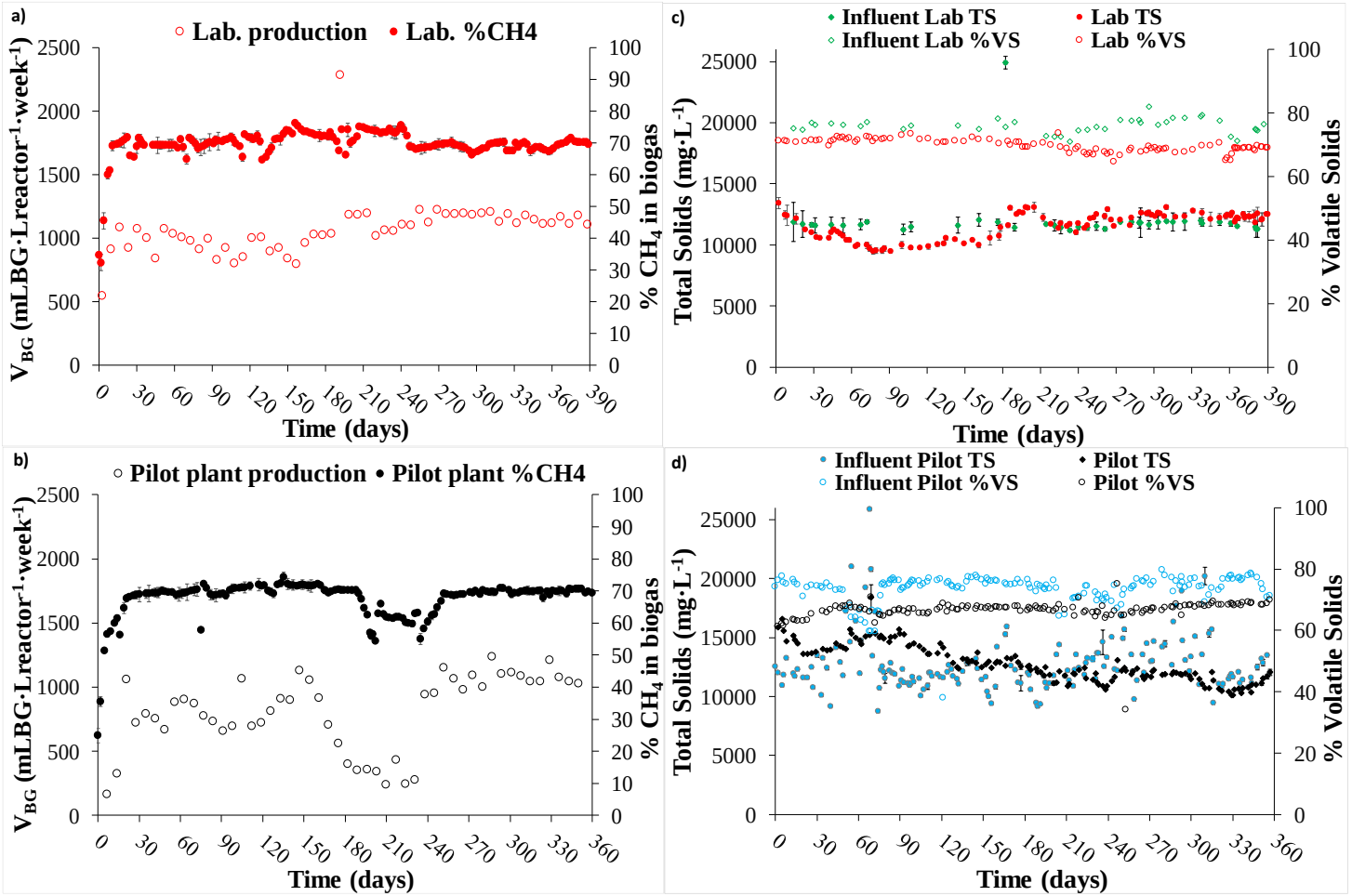

Figure 6.2. Biogas (BG) production and methane (CH_4) percentage at lab-scale (a) and pilot-scale (b); total solids (TS) and volatile solids (VS) percentage evolution at lab-scale (c) and pilot-scale (d).

The average methane yield obtained in the present work is high compared to other microalgae co-digestion studies with sewage sludge working at similar conditions. Olsson et al. (2017) reported a methane yield of 168 $mLCH_4 \cdot gTVS_{inf}^{-1}$ when digesting microalgae with primary and waste activated sludge in a proportion of 40% microalgae and 60% mixture sludge based on TVS in a semi-continuous system. Solé-Bundó et al. (2019) observed a methane yield of 200 $mLCH_4 \cdot gTVS_{inf}^{-1}$ when digesting microalgae (*Chlorella*) as unique substrate and a methane yield of 333 $mLCH_4 \cdot gTVS_{inf}^{-1}$ when co-digesting it with primary sludge (in a proportion of 25/75% based on TVS) in a mesophilic continuous CSTR. The proportions of sludge used in the above-mentioned studies were the same or higher than the one used in the present study so the high values obtained in the present work could be attributed to the AnMBR configuration. Serna-García et al. (2020b) and Greses (2017) reported a methane production improvement and a major microbial community diversity due to the implementation of a membrane in the system.

ACoD led to an effluent rich in nutrients: around 423 $mg \cdot L^{-1}$ of NH_4-N and 25 $mg \cdot L^{-1}$ of PO_4-P were obtained at lab-scale (Table 6.3). Ammonium reached a concentration higher than that present in the influent (Table 6.3), demonstrating the nitrogen mineralization during AD. However, phosphate content (25 $mgPO_4\text{-}P \cdot L^{-1}$) in the effluent was lower than the influent content (36 $mgPO_4\text{-}P \cdot L^{-1}$), probably due to uncontrolled phosphorus precipitation occurring inside the reactor; problem previously reported by other authors (Martí et al., 2017, Sánchez-Ramírez et al., 2019). Nevertheless, the high nitrogen content (Table 6.3) makes the ACoD effluent a product with potential to be used as soil improver.

6.3.1.1. Effect of SRT

Previous laboratory co-digestion experiments (Serna-García et al., 2020a) in the same reactor showed a biodegradability percentage of 73% when digesting *Chlorella* at an SRT of 100 d (keeping the rest of operating conditions equal to the ones used in the present work) (Table 6.3). This 73% percentage is a higher value than the one observed in the present study (65%). This is in accordance with Greses (2017), who studied three different SRT conditions (70, 100 and 140 d), reporting that the higher the SRT, the higher the biodegradation potential, when digesting microalgae in an AnMBR. Then, operating at 100 d SRT should be considered to scale-up the co-digestion process. However, considering the same input flow characteristics and keeping the mixed liquor concentration

in the same range to avoid problems related with membrane operation, operating a co-digestion plant at an SRT of 100 d would require a higher process volume than operating at 70 d and, as a consequence, a higher number of reactors. An economic balance was carried out to consider operation and construction costs and to compare them with the benefits of generating a higher biogas production.

Since the material used for the lab-scale AnMBR construction was polyvinyl chloride (PVC), construction costs are not completely realistic when thinking about the scale-up process. Thus, the economic balance carried out in the present work, which is based on an incoming flow of 1526 $m^3 \cdot h^{-1}$ related to a large-scale WWTP (Cuenca del Carraixet WWTP, 155.674 PE), was performed using concrete as a construction material. A reactor thickness of 0.5 m and a flap thickness of 0.3 m were chosen for calculations. The economic balance showed initial construction costs and annual depreciation costs 43% higher for a reactor operated at 100 d of SRT compared to a reactor operated at 70 d of SRT. If the biogas produced were totally converted to electricity, the profit generated operating a 100 d SRT reactor would be 13% higher than operating at 70 d SRT. Then, annual benefits taking into account the annual depreciation costs and profits obtained from biogas would be 3% higher for operating a reactor at 70 d. The payback period for a 70 d SRT system would be 10.7 years and 15.8 years for a 100 d SRT system. Then, although the digester operation at 100 d of SRT increases the biodegradability from 65% to 73%, the economic results do not suggest working at this SRT value. Thus, an SRT of 70 d was chosen to operate the ACoD pilot plant (Section 6.3.2).

6.3.1.2. Effect of temperature

Previous experiments at lab-scale (Serna-García et al., 2020b) showed a biodegradability percentage of 69% when digesting the same microalgae (*Chlorella*) and primary sludge, working at the same operating conditions but at thermophilic temperature (55 ºC) (Table 6.3). This is a bit higher value compared with the present study (65%). However, the energy demand for operating a thermophilic system is higher compared to mesophilic, so, an energy balance was carried out to choose the best temperature operation for the ACoD pilot plant.

The energy balance was calculated at lab-scale and large-scale. The heating requirements were calculated considering the energy necessary for increasing the influent stream

temperature from 25 ºC to 35 or 55 ºC and for compensating the reactor's heat losses (heat dissipated through the reactor's walls). The heat transfer coefficient (U) at lab-scale had a value of 0.65 kcalh·m^{-2}·K^{-1}. Values for total energy demand (Q_{demand}) at lab-scale were: 128 kWh·m^{-3} and 385 kWh·m^{-3} for operation at 35 and 55 ºC, respectively. These values are too high due to the impossibility of applying a CHP treatment for heat recovery at lab-scale since the treated flowrate was too low. Nevertheless, the energy demand was much higher (three times higher) at thermophilic temperature suggesting the advantage of applying mesophilic temperatures.

As well as for economic balance in Section 6.3.1.1., energy balance was also calculated at large-scale to consider the materials used for reactor construction at this scale and to consider the possibility of applying a CHP treatment for heat recovery. The U had a value of 2.27 kcalh·m^{-2}·K^{-1}. Results from energy balance calculations showed a 5.5-fold increase in energy demand working at thermophilic temperature comparing to mesophilic. Then, a significant amount of energy would be required in the AnMBR for increasing temperature from 35 to 55 ºC, not compensating the increase in methane production that takes place raising the temperature of operation. Thus, 35 ºC was the temperature chosen to operate the ACoD pilot plant (Section 6.3.2).

6.3.1.3. Membrane operation

The AnMBR filtration at laboratory scale was studied for 30 days (from Day 356 of AnMBR operation) and membrane permeability and SMPs concentrations were evaluated. The membrane permeability was stabilised around 35 LMH·bar^{-1}. Despite the low permeability achieved, not significant losses of permeability were detected during the experiment suggesting that negligible irreversible fouling could be expected at short/middle-term. This hypothesis was corroborated by the performed short-term flux analysis evaluation (see Figure 6.3), which shows similar filtration resistances during the conducted study even at relatively high fluxes. The low permeabilities achieved during the filtration process were attributed to the middle/high mixed liquor viscosity (around 1.5 cP) and the low gas sparging employed during the experiment (0.15 N·m^3·m^{-2}·h^{-1}).

The concentration of SMPs in the reactor also remained stable during the 30-days experiment. The proteins and carbohydrates achieved concentrations around 15 and 54 mg·L^{-1}, respectively. Despite the high lipids content presented by both co-substrates,

microalgae and primary sludge (Solé-Bundó et al., 2019; Ullah et al., 2015), an accumulation of SMPs inside the reactor was not observed, so, the use of this biomass for biogas production did not affect negatively on the filtration process in the short/middle-term. Then, filtration process at lab-scale showed stability during the one-month period of study, suggesting that the scale-up of the complete AnMBR layout could be performed with promising results.

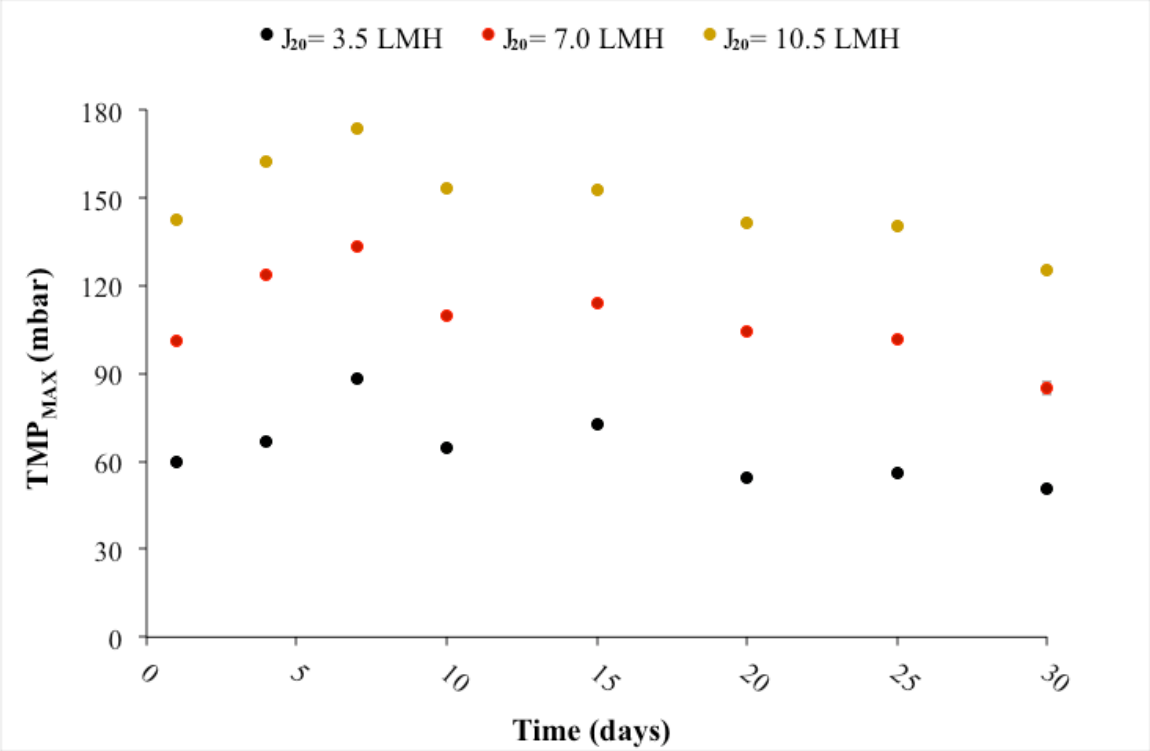

Figure 6.3. Filtration performance in lab-scale operation: transmembrane pressure (TMP) evolution at different permeate fluxes (J_{20}) for irreversible fouling determination.

6.3.2. Pilot-scale

Results shown in Section 6.3.1. are based on a small laboratory system (Figure 6.1a) that was operated in a semi-continuous mode and was not automated, as explained in Section 6.2.2.1. The scale-up of this process from laboratory to pilot-scale is a first approach for future full-scale implementation. In this scale-up step, a system with a higher volume and a more complex equipment was used for co-digestion experiments (Figure 6.1b). Furthermore, the system was operated in a continuous mode and it was totally automated implementing an ICA system. Considering energy and economic balance calculations (Section 6.3.1.), the ACoD pilot plant was operated at 35 ºC and 70 d of SRT. Since

filtration experiment at lab-scale showed stability during 30 d, different filtration conditions were evaluated at pilot-scale to optimise the process for future full-scale application.

6.3.2.1. Process performance

The ACoD pilot plant was operated for 360 days. The process efficiency was evaluated by monitoring several parameters such as biogas production, TS and TVS concentration (Figure 6.2) and nutrients concentration. The TS concentration was reduced from a maximum value of 16560 $mg \cdot L^{-1}$ at the beginning of the experiment to 11450 $mg \cdot L^{-1}$ (Figure 6.2d) when pseudo steady state was reached as a consequence of the high hydrolytic activity of the anaerobic microorganisms. The TVS removal percentage achieved at pilot plant scale was around 64% (Table 6.3). Biogas production started increasing since the beginning of the experiment, but a sharp drop in the production was observed after Day 167 (Figure 6.2b), related to gas leakage. Stability was recovered after 70 d. Once pseudo steady state was reached (from Day 250 onwards), the AnMBR showed a biogas production of 78 $L \cdot d^{-1}$, which corresponds to a methane yield of 215 $mLCH_4 \cdot gCOD_{inf}^{-1}$ (370 $mLCH_4 \cdot gTVS_{inf}^{-1}$). This value is similar to the one observed at laboratory-scale (Table 6.3); 154 mL $biogas \cdot d^{-1} \cdot L^{-1}_{reactor}$ were produced at pilot-scale while 163 mL $biogas \cdot d^{-1} \cdot L^{-1}_{reactor}$ were produced at laboratory-scale (Figure 6.2a). Then, the results from laboratory performance and pilot-scale ACoD are in good agreement.

The harvested microalgae from the membrane photobioreactor pilot plant co-digested in this experiment were mainly composed of *Chlorella*, but a bloom of *Scenedesmus* was observed during the pilot plant operation (from Day 90 to 113), as reported by González-Camejo et al. (2019). Nevertheless, the biogas production remained stable (Figure 6.2b). Other authors have reported different biogas production when digesting these two species (*Scenedesmus* or *Chlorella*) at lab-scale (Frigon et al., 2013; Mussgnug et al., 2010; Greses, 2017). For instance, Greses (2017) observed a higher biodegradability (71%) when digesting *Scenedesmus* compared to *Chlorella* (62%) at mesophilic conditions. In the present study, the change in microalgae species did not affect the AnMBR performance. This is in accordance with a previous microalgae co-digestion study (Serna-García et al., 2020a) at lab-scale, in which no difference in biogas production was observed when microalgae species shifted from *Scenedesmus* to *Chlorella*. Co-digesting microalgae with

primary sludge offers a robust system that is a promising approach for future industrial application.

ACoD at pilot-scale resulted in a nutrient-rich effluent: around 400 $mg \cdot L^{-1}$ of NH_4-N and 37 mg L^{-1} of PO_4-P were obtained, similar values to the ones obtained at lab-scale (Table 6.3). During microalgae and primary sludge ACoD, not only methane-rich biogas was generated but also a nutrient-rich effluent, obtaining two potential products for resource recovery (energy and nutrients) from wastewater.

6.3.2.2. Membrane operation

Short-term trials were conducted before mid-term experiments to determine operating conditions at pilot-scale. The results are shown in Figure 6.4a. With a fixed J_{20} of 5.6 LMH, three different SGDs (0.15, 0.33 and 0.62 $N \cdot m^3 \cdot m^{-2} \cdot h^{-1}$) and two different filtration times (300 s and 160 s) were tested, with a 45 s backwash every filtration cycle. At 300 s of filtration time, SGD seemed to be relevant, but not at 160 s, so higher SGD conditions (0.62 $N \cdot m^3 \cdot m^{-2} \cdot h^{-1}$) were selected for 300 s (Experiment I), and lower SGD conditions (0.15 $N \cdot m^3 \cdot m^{-2} \cdot h^{-1}$) were selected for 160 s time (Experiment II) due to similar short-term TMP performance (Table 6.1).

Results from mid-term ACoD pilot plant filtration trials are shown in Figure 6.4b. Membrane permeability decreased in Experiment I, slowly first and faster after Day 3, reaching values around 85 $LMH \cdot bar^{-1}$ after 6 days of operation. After a few days and a physical cleaning, Experiment II began, showing a fast initial decrease in membrane permeability first day of operation, and a slow decrease in the next few days, reaching values around 24 $LMH \cdot bar^{-1}$. Both Experiments I and II showed a faster decrease in permeability than expected, possibly due to supercritical flux. Experiment III was conducted after a physical cleaning, with a 25% reduction in J_{20}, to 4.2 LMH, 160 s of filtration time and 0.15 $N \cdot m^3 \cdot m^{-2} \cdot h^{-1}$ of SGD (Table 6.1), but including a 30 s relaxation stage and decreasing backwashing frequency to 2 F-R cycles, to compensate lower flux. Experiment III showed a significantly lower membrane permeability decrease than Experiment II, reaching 24 $LMH \cdot bar^{-1}$ after 11 days. Indeed, this permeability-decreasing rate was half of that observed in Experiment II and close to that observed in Experiment I. To test possible enhancement due to SGD increasing effect, Experiment IV was conducted with the same conditions as Experiment III, but increasing SGD to 0.33 $N \cdot m^3 \cdot m^{-2} \cdot h^{-1}$

(Table 6.1). A permeability of 65 LMH·bar^{-1} was reached after 4 days in Experiment IV, showing a slightly lower permeability-decreasing rate than the observed in Experiment III. Then, the permeability decrease rate showed a similar value in Experiments I and IV, stayed in slightly higher but acceptable values in Experiment III and was significantly higher in Experiment II.

Permeability in pilot plant filtration experiments showed similar values to the laboratory ones. Both laboratory and pilot-scale experiments reached similar values to those obtained in other filtration processes with microalgae biomass presence. For instance, Hapońska et al. (2018) found permeabilities between 18.3 and 32.7 LMH·bar^{-1} when harvesting microalgae with dynamic filtration focused to maximize final microalgae sludge concentration at pilot-scale. Compared with permeabilities reached by Kim et al. (2015) in their experiments of high-density microalgae harvesting, results in this work were better than theirs with static ultrafiltration (3.8 – 4.8 LMH·bar^{-1}), but similar to dynamic ultrafiltration at 400 rpm (68 – 75 LMH·bar^{-1}). Results are also in accordance with Zhang et al. (2013), who obtained a permeability of 60 LMH·bar^{-1} when filtrating *Microcystis aeruginosa* with a ceramic microfiltration membrane.

The four experiments carried out at pilot-scale tried to find an optimal way to operate the membrane unit, reducing the power consumption of the biogas blower but maintaining a reasonable permeability. Experiment I showed good permeability, but SGD value was too high. The low SGD in Experiment II led to an excessive permeability reduction. Experiment III was conducted with a 25% lower J_{20} (compared to Experiment II), not only compensating the reduction of backwash frequency but also improving permeability. SGD in Experiment IV was doubled compared with Experiment III, but permeability was slightly improved, so the increase of power consumption was not justified. In consequence, Experiment III conditions seem to be preferable for operating an ACoD pilot plant filtration process with microalgae and primary sludge as co-substrates.

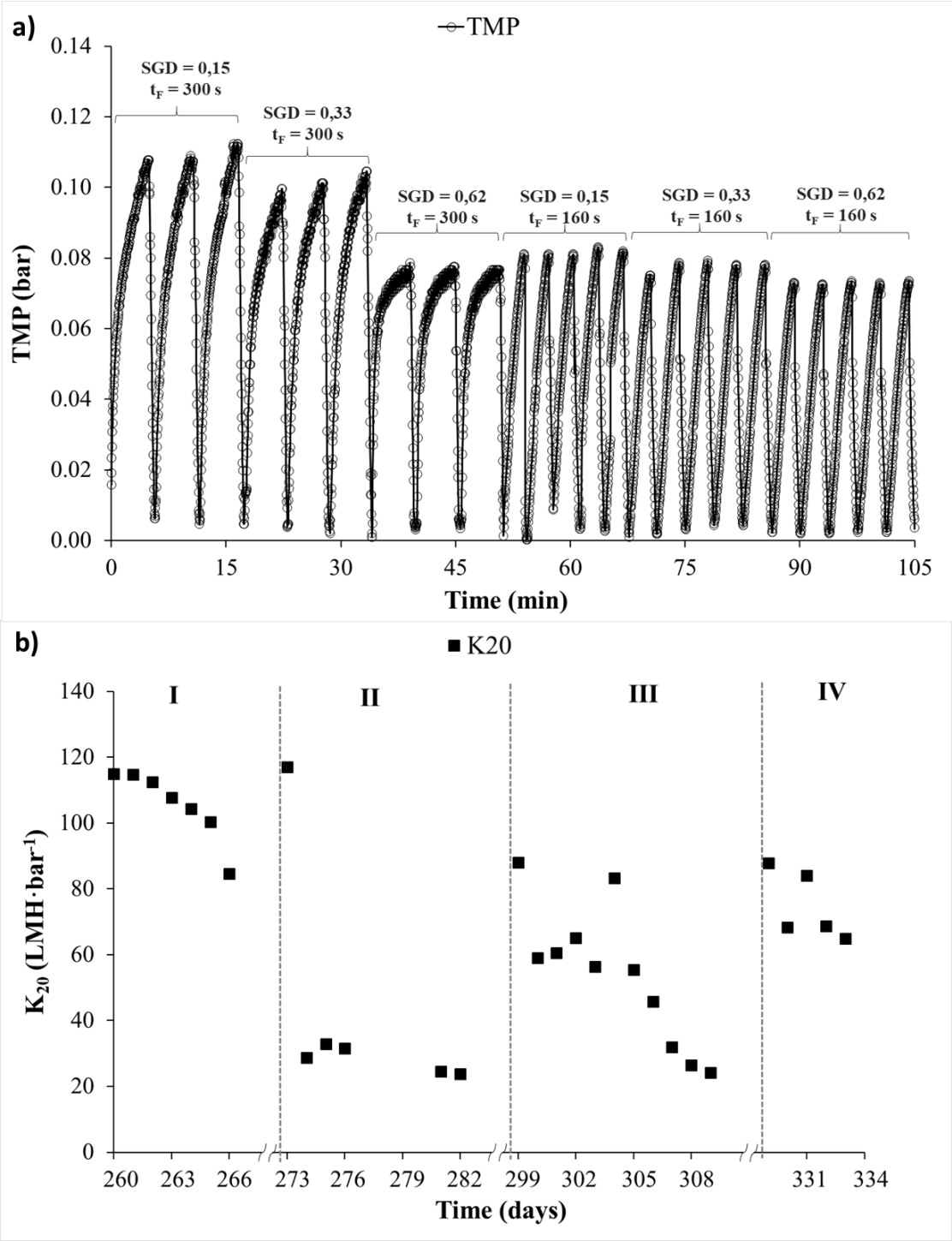

Figure 6.4. Anaerobic co-digestion pilot plant short-term filtration trials (a) and permeability evolution during the four experimental periods at mid-term filtration trials (b). SGD: specific gas demand; TMP: transmembrane pressure; t_F: filtration time; K_{20}: membrane permeability.

Findings from the present study indicate that the results obtained at a small scale (laboratory) working with real substrates (microalgae and primary sludge) could predict the results obtained at pilot-scale. Both the biological and filtration processes showed a good performance, suggesting that microalgae and primary sludge ACoD in an AnMBR is a promising approach for future full-scale application, recovering valuable resources (energy and nutrients) from sewage. However, even data from economic and energetic balance regarding co-digestion operation have been provided to compare different SRTs and temperatures to choose the best operating conditions for the ACoD pilot plant, a deeper economic analysis and a life cycle assessment need to be considered for future industrial-scale implementation.

6.4. CONCLUSIONS

- ACoD operation at 35 ºC and 70 d SRT was selected as the optimal conditions applying an energy and economic balance.
- Primary sludge and microalgae ACoD at lab-scale resulted in 163 mLbiogas·d^{-1}·$L^{-1}_{reactor}$ leading to high methane yield (391 mLCH_4·gTVS_{inf}^{-1}).
- One-year operation at pilot-scale at the selected conditions showed methane yield of 370 mLCH_4·gTVS_{inf}^{-1} corroborating the results previously obtained at lab-scale.
- Filtration showed stable performance at laboratory and pilot-scale working at J_{20} of 4.6-5.8 L·m^{-2}·h^{-1}. Applying SGD (0.15 N·m^3·m^{-2}·h^{-1}) and conducting a backwash cycle every two filtration cycles was enough for controlling fouling formation.

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

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Supplementary information Chapter VI

Table 6.S1. Considerations for energy and investment balance at different scales.

	Lab-scale	Pilot-scale	Industrial-scale
Input flow ($m^3 \cdot d^{-1}$)	0.00046	0.03	2014
Reactor thickness (m)	0.05	0.15	0.5
Reactor height (m)	0.4	2.2	13
Reactor diameter (m)	0.19	1	26
$K_{reactor}$ ($kcal \cdot h^{-1} \cdot m^{-1} \cdot K^{-1}$)	0.03	0.03	1.40
Energy (€·kWh^{-1})	-	-	0.11*
Land cost (€·m^{-2})			1.02**
Material for construction	PVC	PVC	Concrete
Concrete wall (€·m^{-3})	-	-	350
Concrete slab (€·m^{-3})	-	-	130

$K_{reactor}$: reactor material conductivity; PVC: polyvinyl chloride
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Table 6.S2. Construction and maintenance expenses for an industrial anaerobic co-digestion plant.

	70 d SRT	100 d SRT
Volume required (m^3)	141006	201437
Construction costs (k€)	4569	6528
Land surface (k€)	44	63
Equipment* (k€)	5503	7862
Total investment costs (k€)	10116	14453
Annual depreciation costs (k€)	505	722
Annual energy generated from biogas (k€)	1443	1635
Annual benefits (k€)	938	913

SRT: solids retention time
**Pumps and blowers*

Maximising resource recovery from wastewater grown microalgae and primary sludge in an anaerobic co-digestion plant coupled to a composting process

7. MAXIMISING RESOURCE RECOVERY FROM WASTEWATER GROWN MICROALGAE AND PRIMARY SLUDGE IN AN ANAEROBIC MEMBRANE CO-DIGESTION PILOT PLANT COUPLED TO A COMPOSTING PROCESS

Abstract: An anaerobic membrane bioreactor (AnMBR) was run during one year for microalgae (*Chlorella* spp.) and primary sludge anaerobic co-digestion (ACoD) at pilot-scale. The AnMBR was operated at 35 ºC, 70 d of solids retention time and 30 d of hydraulic retention time, showing a high stability in terms of pH and VFA concentration. A methane yield of 370 $mLCH_4 \cdot gVS_{inf}^{-1}$ was observed, demonstrating the high degradation of microalgae and primary sludge substrates. Nutrient-rich effluent streams (685 $mgN \cdot L^{-1}$ and 145 $mgP \cdot L^{-1}$ in digestate and 395 $mgNH_4\text{-}N \cdot L^{-1}$ and 37 $mgPO_4\text{-}P \cdot L^{-1}$ in permeate) were obtained, allowing posterior nutrients recovery. Ammonium from permeate was recovered as ammonia sulphate through a hydrophobic polypropylene hollow fibre membrane contactor, resulting in a 99% efficiency in nitrogen recovery. However, phosphorus recovery through processes such as struvite precipitation was not applied since only 26% of phosphate was available in the effluent. Composting process assessment of digestate coming from the ACoD pilot plant was carried out at laboratory-scale with Dewar reactors. Composting of conventional sludge coming from an industrial WWTP digestion process was also assessed to compare results, observing similar values with both sludges studied. A final composted material, sanitised (free of *Escherichia coli* and *Salmonella* spp.) and stable (respirometric index at 37 ºC was below 0.5 $mgO_2 \cdot g$ organic $matter^{-1} \cdot h^{-1}$) was obtained for both sludges.

Keywords: composting; anaerobic co-digestion; microalgae; resource recovery; nutrients; methane

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7.1. INTRODUCTION

Classical Wastewater Treatment Facilities (WWTF) in which waste is derived from the purification process are now being replaced by new Water Resource Recovery Facilities (WRRF) in which waste is re-used to generate products of agronomic and commercial interest instead of being simply managed. Research in the 21st century is focused on wastewater management through anaerobic digestion (AD) processes, which, combined with membrane separation technologies is a promising approach that is generating a growing interest in scientific community. Indeed, anaerobic membrane bioreactors (AnMBRs) are being applied for wastewater treatment for their several advantages, which allow a higher resource recovery from wastewater, as well as a decrease in energetic costs in comparison with the biological aerobic conventional systems (Becker et al., 2017; Dereli et al., 2012; Giménez et al., 2011; Robles et al., 2018).

Autotrophic microalgae-based technology is also being used for nutrient removal from the waterline (Acién et al., 2016; González-Camejo et al., 2020; Khalid et al., 2019). Microalgae are able to hold back a higher nutrient concentration than conventional treatments and generate better clarified effluent and sludge with a higher concentration of ammonium and phosphate (Acién et al., 2016; González-Camejo et al., 2020). Wastewater-grown microalgae biomass can be harvested and used as a substrate for AD. Indeed, microalgae biomass AD generated in a membrane photobioreactor pilot plant (MPBR) has been reported to be efficient in terms of methane production (Greses et al., 2017) on a laboratory scale. However, this efficiency could be improved by digesting the microalgae biomass with primary sludge as a co-substrate (Serna-García et al., 2020a; Solé-Bundó et al., 2019). These authors obtained better biodegradability percentage when digesting microalgae and primary sludge on a lab-scale comparing to microalgae AD as unique substrate. However, this process needs to be evaluated at pilot-scale as a first step for future industrial application.

In this sustainable scheme for wastewater treatment, AD also has a nutrient recovery potential. Traditional wastewater treatment plants (WWTPs) used to remove nitrogen (N) from the effluent through a biological nitrification/denitrification step and phosphorus (P) through enhanced biological P removal or chemical precipitation. However, there are other techniques that make nutrient recovery from AnMBR effluents a feasible option for

a circular economy-based scenario. Although struvite precipitation is a useful alternative for recovering both P and N, at least 50 ppm of phosphate (PO_4-P) are needed to make it profitable (Cornel et al., 2009). Modifications have thus been proposed in the WWTP layout to increase this PO_4-P concentration in AD (Martí et al., 2008). Although high P recovery efficiencies (80-90%) can be obtained through struvite precipitation, N recovery is not highly efficient (20-30%) and other technologies can be used such as bioelectrochemical systems, electrodialysis or hollow-fibre membrane contactors (HFMC). HFMC appears to be an interesting treatment because of its low volume and energy requirements. In these systems, free ammonia nitrogen (FAN) passes through a microporous hydrophobic membrane and a sulphuric acid solution is used as the draw solution to recover N as valuable ammonia sulphate.

As not only nutrient-rich permeate, but also nutrient-rich digestate is obtained from AnMBR processes (Nag et al., 2019, Nkoa, 2013; Seco et al., 2018), the digestate has potential agricultural applications since it could be used as a fertiliser. However, direct land application of digestate presents some drawbacks: i) large agricultural areas are needed to directly apply the large amount of digestate generated in AD plants, involving high transport costs (Fuchs and Drosg, 2013); ii) in some cases, especially when digesting substrates with high slowly biodegradable volatile solids (VS) content, such as microalgae, the digestate still contains undigested VS and needs further stabilization; (iii) the possible presence of pathogens or heavy metals (Monlau et al., 2015). These drawbacks lead to the necessity of a forward stabilisation process to produce stable organic soil improver.

Composting has been shown to be an effective process for treating different organic wastes including anaerobic digestate, municipal solid wastes and manure wastes, among others. However, the composting process of an anaerobic digestate from a microalgae and primary sludge co-digestion plant has not yet been evaluated, to the best of the authors' knowledge. In a composting process, the biological decomposition of organic waste takes place under controlled aerobic conditions, involving mesophilic and thermophilic microorganisms. Organic substrates are transformed into a stabilised material free of pathogens and ready to be used in agriculture. This process depends not only on environmental factors such as pH, aeration, moisture content or temperature, but also on sludge characteristics such as nutrient content, particle size or carbon to nitrogen (C/N)

ratio (Nikaeen et al., 2015). The C/N ratio is one of the most important parameters of the composting process (Gao et al., 2010; Puyuelo et al., 2011) since is used as an initial requirement to provide the optimum conditions for development of microorganisms and, as a monitoring parameter. Due to the low C/N ratio of anaerobic digestate, especially when treating substrates with high N content such as microalgae or sludge (Solé-Bundó et al., 2019; Ullah et al., 2015), a bulking agent (BA) can be added to generate a mixture with an appropriate C/N ratio (20-25) (Huang et al., 2004).

There are a wide variety of microorganisms in a composting system, the most abundant being fungi, actinomycetes and bacteria (Silva and Naik, 2007). According to the Spanish Regulation RD 506/2013 (Annex IV), compost is required to contain less than 1000 most probable number (MPN) of *Escherichia coli* (*E. coli)* per gram of final product and *Salmonella* spp. has to be absent in 25 grams.

In this work the long-term anaerobic co-digestion (ACoD) of raw microalgae biomass and primary sludge was evaluated at pilot-scale. Potential nutrient recovery (P and N) from the ACoD permeate through struvite crystallization and HFMC was evaluated. A laboratory-scale composting process of the ACoD sludge was also applied. Composting parameters that ensured the generation of a stable organic improver were assessed. ACoD sludge composting was compared to a conventional sludge composting process to determine whether the presence of microalgae substrate in the digestate influenced the composting process in this first study to evaluate complete resource recovery from microalgae and primary sludge co-digestion.

7.2. METHODS AND MATERIALS

7.2.1. Anaerobic co-digestion pilot plant description

An ACoD pilot plant located in Cuenca del Carraixet' WWTP (Valencia, Spain) was used for the ACoD experiments. This plant consisted of an anaerobic digester, with a total working volume of 900 L coupled to a 1-L membrane tank fitted with a 0.42 m^2 hollow fibre ultrafiltration membrane unit (0.03 µm pores, PURON® KMS, USA) (Figure 7.1a). The pilot plant was operated for one year at a solids retention time (SRT) of 70 d, while hydraulic retention time (HRT) was set at 30 d (an extensive description of the ACoD pilot plant can be found in Serna-García et al., 2020b). The ACoD pilot plant feedstock

was a mixture of microalgae biomass, cultivated in a MPBR plant (González-Camejo et al., 2017) and primary sludge coming from the same WWTP's thickener. These both substrates were fed in proportions according to the results from previous laboratory works (Serna-García et al., 2020a): 62% primary sludge and 38% microalgae based on VS content. The substrates were diluted previous to the reactor's feeding until achieving the desired organic loading rate (OLR): 0.5 $gCOD \cdot d^{-1} \cdot L^{-1}$ and mixed in an equalisation tank.

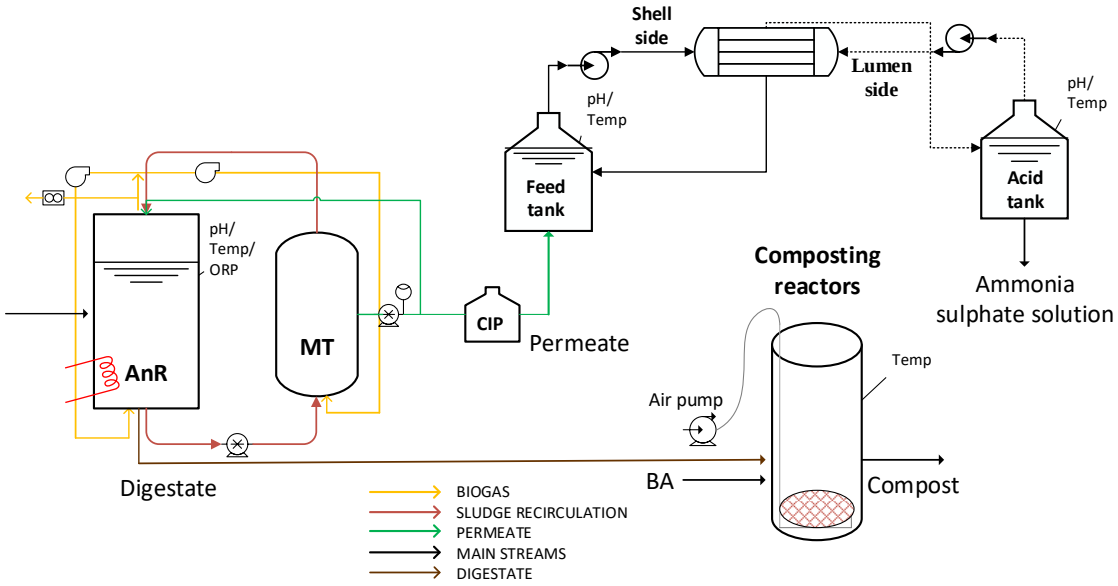

Figure 7.1. Layout of the experimental set-up: anaerobic co-digestion pilot plant (a), HFMC set-up (b) and an example of composting reactor (c). AnR: anaerobic reactor, MT: membrane tank, CIP: clean-in-place tank; BA: bulking agent.

7.2.2. HFMC set-up

A hydrophobic polypropylene HFMC (X50 2.5x8 Liqui-Cel®, USA) with a surface of 1.4 m^2 was used for N recovery at laboratory-scale. Two closed tanks of 1.2 L were used to store the permeate and acid solution (Figure 7.1b). Each tank was equipped with pH and temperature electronic sensors (SP10T, Consort®, Belgium) connected to a multiparametric analyser (Consort® C832, Belgium). The acid stream (0.05M H_2SO_4) circulated in the lumen side at a flow rate of 0.4 $L \cdot min^{-1}$ while the permeate was fed to the shell side at a flow rate of 0.6 $L \cdot min^{-1}$ and a pH of 10. Since the different ammonia (NH_3) concentrations on each side of the membrane are the driving force, it is necessary to work at pH over 8.6 in the feeding solution so, sodium hydroxide (1M) was used to

adjust the pH. Both streams were recirculated and fed counter-currently. Filtration and settling were applied as pre-treatment to avoid membrane clogging.

7.2.3. Composting experiments

7.2.3.1. Reactors description

Seven cylindrical composting reactors (R_{A1}-R_{A4} and R_{C1}-R_{C3}) were operated at lab-scale. The composting reactors were 4 L glass Dewar flasks (KGW Isotherm, Germany) covered by an aluminium coating with cork insulation between the inside and the outside wall, offering a high thermal isolation. A plastic mesh was incorporated at the bottom of all the reactors covered by a gravel layer to allow a correct separation between leachate and composted material. The Dewar flask covers were perforated to allow gas evacuation. A layout of the composting reactor is shown in Figure 7.1c.

7.2.3.2. Composting substrates

Two types of sludge were used to generate the mixtures in each composting reactor. The first, henceforward called 'ACoD sludge', was obtained from the ACoD pilot plant described in Section 7.2.1. The second, henceforward called 'conventional sludge', was an anaerobic sewage sludge from the Carraixet WWTP conventional AD process, operated at an SRT of 20 d. Conventional sludge was used as reference substrate to compare the composting results obtained with the ACoD sludge under study. Both sewage sludges were pre-treated in a centrifuge to remove excess moisture, achieving values around 80-87% of moisture. A cationic polyelectrolyte was added to conventional sludge that had been dried in the industrial centrifuge of the Carraixet WWTP. ACoD sludge was dried in a lab-scale centrifuge (Eppendorf, Centrifuge 5804) at 4350 rpm for 30 minutes and the obtained pellet was centrifuged for a further 15 minutes. The ACoD sludge was left to air-dry for 48 hours before its use.

Five different BA were characterised (Table 7.1). Regarding the analytical results of each BA and its availability for being collected at University of Valencia´s garden, pruning remains were chosen as BA. These remains were shredded to achieve the correct size for assimilation by microorganisms involved in the process.

Table 7.1. Bulking agent characterisation.

Bulking agent	C/N ratio	Nitrogen (%)	Moisture (%)
Pruning remains	49.5 ± 0.2	0.90 ± 0.00	49.1 ± 0.4
Lawn	13.9 ± 0.5	3.08 ± 0.09	78.9 ± 0.8
Olive wood	109 ± 1	0.42 ± 0.02	48.3 ± 1.1
Cypress tree	58.4 ± 0.3	0.81 ± 0.01	57.9 ± 0.2
Orange tree	29.4 ± 0.9	1.50 ± 0.02	30.6 ± 0.6

7.2.3.1. Experimental design

Reactors were operated around a maximum of 44 days, in pairs, to evaluate the effect of different operating conditions on the composting process. Three parameters were assessed: i) aeration, ii) addition of inoculum and iii) mixture of sludge and BA. Table 7.2 shows the main operating conditions of the seven reactors. Reactors R_{A1} to R_{A4} were fed with ACoD sludge while reactors R_{C1} to R_{C3} were fed with conventional sludge.

The effect of aeration was evaluated operating the pairs of reactors R_{A1} and R_{A3} and R_{C1} and R_{C3}, which were run under the same operating conditions but changing aeration mode: R_{A1} and R_{C1} were operated with forced aeration while R_{A3} and R_{C3} were turned by hand (Table 7.2). The effect of adding inoculum to the reactor was assessed operating the pair of reactors R_{A2} and R_{A4}, which had the same operating conditions but reactor R_{A2} had inoculum while reactor R_{A4} had not (Table 7.2). Finally, the effect of mixing different proportions of sludge with BA was evaluated operating pairs of reactors R_{A2} and R_{A3} and R_{C2} and R_{C3}, which were run under the same operating conditions but with different proportions of sludge and BA (Table 7.2). Proportions of each mixture´s component were calculated as 'volumetric proportions' and 'theoretical proportions´. Volumetric proportions were generated with 2.5 volumes of BA per each volume of sludge, a common ratio used in sludge composting facilities. Theoretical proportions were calculated using Eq.1, in which the weight of each mixture's component is determined by setting the C/N ratio in a value of 25 and the moisture content in a value between 50% and 70%.

$$\frac{m_{BA}}{m_S} = \frac{(25 \cdot N_S(kg)) - C_S(kg)}{C_{BA}(kg) - (25 \cdot N_{BA}(kg))} \quad \text{(Eq. 7.1)}$$

m_{BA} and m_S being proportions of BA and sludge, respectively; N_S and C_S the sludge N and carbon content, respectively; and N_{BA} and C_{BA} the BA N and carbon content, respectively.

Table 7.2. Main operating conditions of each composting reactor.

Reactors identification	Days in operation	Sludge	Mixture proportions	Aeration	Inoculum
R_{A1}	36	ACoD	Theoretical	Forced	✓
R_{C1}	36	Conventional	Theoretical	Forced	✓
R_{A2}	44	ACoD	Volumetric	Turned by hand	✓
R_{C2}	44	Conventional	Volumetric	Turned by hand	✓
R_{A3}	27	ACoD	Theoretical	Turned by hand	✓
R_{C3}	27	Conventional	Theoretical	Turned by hand	✓
R_{A4}	31	ACoD	Volumetric	Turned by hand	-

ACoD: anaerobic co-digestion; A: ACoD sludge; C: conventional sludge

Temperature was measured daily by a temperature probe inside the reactor. R_{A1} and R_{C1} were adapted to receive forced aeration with a flow rate of 2 $L \cdot min^{-1}$ (by air supplied from the compressed air network) from the bottom of the reactors to the top. The rest of the reactors were manually turned over once a day. In all the reactors, except R_{A4} (Table 7.2), 400 mL of inoculum from the maturation stage of a compost pile from the Vintena composting plant (Carcaixent, Valencia) were added to the mixture inside the reactor to accelerate the speed reaction of the microorganisms involved in the process.

7.2.4. Performance indicators

The ACoD process efficiency was evaluated in terms of biodegradability percentage, biomethane potential and methane yield according to equations previously reported in Serna-García et al. (2020a).

To assess nutrient recovery from the ACoD permeate, P and N recovery were calculated using Eq. 2 and Eq. 3, respectively.

$$\%\ P\ recovery = \frac{P_{eff}}{P_{inf} + P_{rel}} \cdot 100 \quad \text{(Eq. 7.2)}$$

P_{eff} (mgP-$PO_4 \cdot L^{-1}$) being the phosphate concentration in the effluent, $P_{inf,}$ (mgP-$PO_4 \cdot L^{-1}$) the phosphate concentration in the influent and P_{rel} (mgP-$PO_4 \cdot L^{-1}$) the phosphate released into the reactor during AD. This released phosphate is calculated as the influent stream phosphate degraded during AD, according to substrates biodegradability obtained, as will be further explained in Section 7.3.2.

$$\%\ N\ recovery\ efficiency = \frac{TAN_{eff,0} - TAN_{eff,end}}{TAN_{eff,0}} \cdot 100 \quad \text{(Eq. 7.3)}$$

$TAN_{eff,0}$ (mgN-$NH_4 \cdot L^{-1}$) being the initial concentration of Total Ammonia Nitrogen (TAN) in the HFMC influent (permeate from ACoD pilot plant) and $TAN_{eff,end,}$ (mgN-$NH_4 \cdot L^{-1}$) the TAN concentration at the end of the process (HFMC effluent).

C/N ratio, porosity and total nitrogen (TN) of both conventional and ACoD sludge mixtures with BA were measured in the initial composting samples. The presence of pathogens (*E. coli* and *Salmonella* spp.) was measured in both sludges (before being mixed with BA). Moisture and organic matter content, pH and electric conductivity were monitored weekly in the composting reactor samples to assess the process performance. The C/N ratio and pathogens were also analysed in each final product mixture.

According to Barrena et al. (2005), respirometric assays at the in situ temperature are suitable to monitor process biological activity since they are representative of the metabolic state of the microorganisms in the reactor. Nevertheless, assays at 37 ºC are more useful to study the stability of the process. To monitor the biological activity of the composting material, several static respirometric assays at process temperature were therefore performed during the composting process in the reactors that achieved thermophilic temperatures. Static respirometric assays at a fixed temperature of 37 ºC were also performed in the same reactors to analyse the stability of the mixture. The slope of the oxygen concentration (%) versus time curves was calculated for each assay to determine the respirometric index (RI). Respirometric assays and RI calculation were carried out according to Barrena et al. (2005).

7.2.5. Analytical Methods

Total solids (TS), VS, TSS (total suspended solids), VSS (volatile suspended solids), total chemical oxygen demand (TCOD), soluble COD, nutrients concentration (ammonium (NH_4-N), TN, PO_4-P and total phosphorus (TP)), Alkalinity (Alk) and Volatile Fatty Acids (VFA) were measured in triplicate thrice a week according to APHA (2012) procedures. Methane content in the biogas produced was also determined thrice a week using a gas chromatograph equipped with a Flame Ionisation Detector (GC-FID, Agilent Technologies, USA). 1 mL of biogas was collected from the top of the reactor by a gas-tight syringe and injected into a 15 m × 0.53 mm × 1 μm TRACER column (Teknokroma, Spain) which was operated at 40 °C. Helium was the carrier gas at a flow-rate of 40 $mL \cdot min^{-1}$. Methane pure gas (99.99%) was used as standard.

Moisture and organic matter content, pH and electric conductivity were measured according to Standard Methods (APHA 2012) with the corresponding dilutions for adapting the method procedure to compost samples. For instance, for electric conductivity and pH determination, the sample was previously diluted in a ratio of 1:10. The supernatant was analysed after 30 min of agitation and 20 min of centrifugation (11000 rmp).

C/N ratio was determined by measuring the elemental components of the mixture on an Elemental Analyser EA 1110 CHNS (CE Instruments Ltd, Wigan, United Kingdom). A previous pre-treatment of the sample, which consisted of drying the sample at 65 ºC in an oven and applying a milling process, was carried out to transform the heterogeneous material into a homogenous powder. Porosity was determined by the weight difference between the original sample and the sample saturated with water. TN in composting samples was determined according to APHA (2012) with previous homogenisation of the sample in a sonicator (S250D, Branson) and subsequent dilution at a ratio 1:1000.

E. coli presence was quantitatively determined by the standard method for enumeration of *E. coli* β-glucuronidase positive, following the UNE-EN ISO 9308-1:2014. *Salmonella* spp. was measured following the UNE-EN ISO 19250 standard method.

7.3. RESULTS AND DISCUSSION

7.3.1. Anaerobic co-digestion pilot plant performance

Continuous ACoD of microalgae biomass and primary sludge was monitored for one year at pilot-scale. Microscopic study showed that microalgae biomass consisted primarily of *Chlorella* spp. Pseudo steady state was achieved in terms of biogas production, TS and nutrient concentration after 160 days of operation and was maintained and studied for a further period of 200 days. Table 7.3 shows the influent and mixed liquor streams characterisation from the ACoD pilot plant during pseudo steady state. The co-digestion reactor achieved a biogas production of 78 $L \cdot d^{-1}$, with a methane percentage around 69%. Then, a high methane yield of 218 $mLCH_4 \cdot gCOD_{inf}^{-1}$ (371 $mLCH_4 \cdot gVS_{inf}^{-1}$) was obtained compared to numerous studies of microalgae digestion as the sole substrate at laboratory-scale (González-Fernández et al., 2012; Greses et al., 2018; Ras et al., 2011, Wang et al., 2016) even when a pre-treatment was applied to the microalgae biomass (Magdalena et al., 2018; Passos et al., 2014; Solé-Bundó et al., 2018, Wang et al., 2016). This methane yield corresponds to a total biodegradability percentage of 62.5% with a biomethane potential of 61.5%, which indicated that only 1% of the biodegradable organic matter was consumed by sulphate-reducing bacteria. The high AnMBR biodegradation efficiency resulted also in a high COD and VS removal of 63 and 64%, respectively. The system showed high stability since high alkalinity and no VFA accumulation was observed (Table 7.3), which resulted in a stable non-controlled pH during the whole operation. Regarding membrane performance, pilot plant filtration was carried out at an average J_{20} of 4.5 LMH and a filtration time of 180 s, showing stability. No membrane replacement was needed during the experiment, since applying a specific gas demand of 0.15 $N \cdot m^3 \cdot m^{-2} \cdot h^{-1}$, a backwash cycle every two filtration cycles and physical cleaning was enough to control fouling formation (Serna-García et al., 2020b).

High biogas production from microalgae and primary sludge was observed at long-term pilot-scale operation without the need to apply costly pre-treatments to improve microalgae degradation. This biogas was rich in methane, being a renewable fuel that could be used for energy and heat generation allowing an approach to circular economy scenarios in which a WRRF would be self-sufficient in terms of energy (further research is needed).

Table 7.3. Anaerobic co-digestion (ACoD) pilot plant influent and mixed liquor characterisation during pseudo steady state (mean ± standard deviation values).

	Anaerobic co-digestion pilot plant
Influent	
TS ($mgTS \cdot L^{-1}$)	13086 ± 2009
VS ($mgVS \cdot L^{-1}$)	9919 ± 1592
TSS ($mgTSS \cdot L^{-1}$)	11558 ± 1323
VSS ($mgVSS \cdot L^{-1}$)	8800 ± 970
COD ($mgCOD \cdot L^{-1}$)	15895 ± 1682
TN ($mgN \cdot L^{-1}$)	578 ± 113
NH_4-N ($mgN \cdot L^{-1}$)	97 ± 20
TP ($mgP \cdot L^{-1}$)	137 ± 17
PO_4-P ($mgP \cdot L^{-1}$)	48.0 ± 14.1
SO_4-S ($mgS \cdot L^{-1}$)	194 ± 63
Mixed liquor	
TS ($mgTS \cdot L^{-1}$)	11337 ± 664
VS ($mgVS \cdot L^{-1}$)	7680 ± 457
TN ($mgN \cdot L^{-1}$)	685 ± 80
NH_4-N ($mgN \cdot L^{-1}$)	397 ± 33
TP ($mgP \cdot L^{-1}$)	145 ± 16
PO_4-P ($mgP \cdot L^{-1}$)	36.6 ± 6.1
pH	7.2 ± 0.1
VFA ($mgCH_3COOH \cdot L^{-1}$)	13.1 ± 15.4
Alk ($mgCaCO_3 \cdot L^{-1}$)	2058 ± 109

TS: total solids; VS: volatile solids, TSS: total suspended solids; VSS: volatile suspended solids; COD: chemical oxygen demand, TN: total nitrogen; NH_4-N: ammonium; TP: total phosphorus; PO_4-P: phosphate; SO_4-S: sulphate; VFA: volatile fatty acids; Alk: alkalinity.

7.3.2. Nutrient recovery

ACoD released N to the soluble phase, as expected; around 400 $mgNH_4\text{-}N \cdot L^{-1}$ was present after the ACoD process (Table 7.3). Ammonium remained stable at a concentration higher

than that present in the influent (Table 7.3). Unlike ammonium, phosphate content (37 $mgPO_4\text{-}P \cdot L^{-1}$) in the permeate was lower than the influent content (48 $mgPO_4\text{-}P \cdot L^{-1}$). These results indicated that uncontrolled phosphorus precipitation processes were taking place inside the reactor. This fact has been already observed by several authors (Barat et al., 2009; Doyle and Parsons, 2002; Martí et al., 2017), who reported precipitation problems in the digestion stage of a WWTP when treating sludges coming from biological removal processes. A mass P balance was thus applied to the anaerobic digester considering the average influent and effluent concentrations to estimate the potential P-recovery. P balances were based on the organic P (P_{org}) content per gram of VSS ($gP_{org} \cdot gVSS^{-1}$) in microalgae and primary sludge substrates. This P_{org} content was calculated as the difference between total P concentration and phosphate concentration in each substrate. The experimental values showed a content of 0.010 $gP_{org} \cdot gVSS^{-1}$ for primary sludge and a content of 0.013 $gP_{org} \cdot gVSS^{-1}$ for microalgae. These values were in agreement with those observed in literature: 0.013 $gP_{org} \cdot gVSS^{-1}$ in primary sludge were reported by Marti et al. (2008) and 0.011 $gP_{org} \cdot gVSS^{-1}$ in microalgae were reported by González-Camejo et al. (2020). Two mass balances were carried out (Table 7.4) according to the value of biodegradability used for the calculations. The first balance considered the biodegradability percentage described in Section 7.3.1 (62.5%) obtained digesting microalgae and primary sludge substrates together (combined biodegradability). The second balance was calculated according to the biodegradability percentage obtained when digesting each substrate alone (separated biodegradability), which was 54% for microalgae digestion and 55% for primary sludge (data not shown). Both mass balances showed similar results, revealing meaningful phosphate precipitation (Table 7.4). Around 1.7 $gP \cdot d^{-1}$ were fixed in the reactor representing 74% of the available phosphorus. Only 26% of the phosphate was available for recovery in the effluent (Table 7.4). Influent and effluent calcium and magnesium concentrations (data not shown) indicated a calcium and magnesium precipitation of around 11 and 7%, respectively. This cation precipitation along with the high ammonium concentration suggests the formation of struvite or other phosphate compounds inside the reactor. Then, uncontrolled P precipitation hindered the recovery of phosphate through a struvite precipitation process after the AD step, reducing potential P recovery in the treatment plant. Nevertheless, a significant proportion (145 mg $P \cdot L^{-1}$) was recovered in the biosolids fraction.

Table 7.4. Mass phosphate balances carried out in the anaerobic digester using separated and combined biodegradability. Average values and standard deviation are shown.

	Separated BD	**Combined BD**
$gP_{org} \cdot gVSS^{-1}_{influent}$	0.011 ± 0.007	0.011 ± 0.007
P_{loss} ($gP \cdot kg\ sludge^{-1}$)	7.3 ± 0.7	7.4 ± 0.7
$P_{available}$ ($gP \cdot kg\ sludge^{-1}$)	2.7 ± 0.6	2.5 ± 0.6
Potential $P_{recovery}$ (%)	27.4 ± 6.5	25.5 ± 6.1

BD: biodegradability P_{loss}: phosphorus precipitated; $P_{available}$: phosphorus available for recovery.

Although there was not enough P in the ACoD plant permeate to apply struvite precipitation, the TAN content could be recovered from the permeate. N recovery processes are usually applied after P recovery, mainly struvite precipitation, in which pH is raised and some cations and P precipitate. This precipitation process leads to non-settable solids formation (fine carbonate and phosphate precipitated particles), which must be separated in order to avoid membrane clogging. In this case, as no struvite precipitation step was performed, pre-treatment was needed at the beginning of the process to raise the pH and later separate the solids formed. In these steps some N (around 15% w/w) was lost by stripping, which is a similar value to that reported by Noriega-Hevia et al. (2020). The results obtained applying HFMC to the ACoD permeate showed a recovery efficiency of 99% in an operating time of approximately 40 min. Figure 7.2 shows the TAN evolution during the experiment. TAN concentration first dropped by 40% after 5 min, reaching the maximum recovery rate because of the high concentration of FAN in the ACoD permeate. As the flux is closely related to the FAN concentration, which decreased, the recovery rate was slowly reduced until complete TAN recovery. Due to the FAN passing through the membrane, the pH in the feed solution storage tank decreased, so that during the experiment the pH had to be maintained at a value of around 10 by adding sodium hydroxide in order to maintain all TAN as FAN and consequently the driving force. The product obtained at the end of the experimentation was an ammonia sulphate solution with a maximum N richness of 4%, which is similar to the obtained by Richter et al., (2019) at full-scale. This ammonia sulphate solution is an inorganic salt that could be used as a commercial fertiliser, being a substitute for currently used chemical fertilisers.

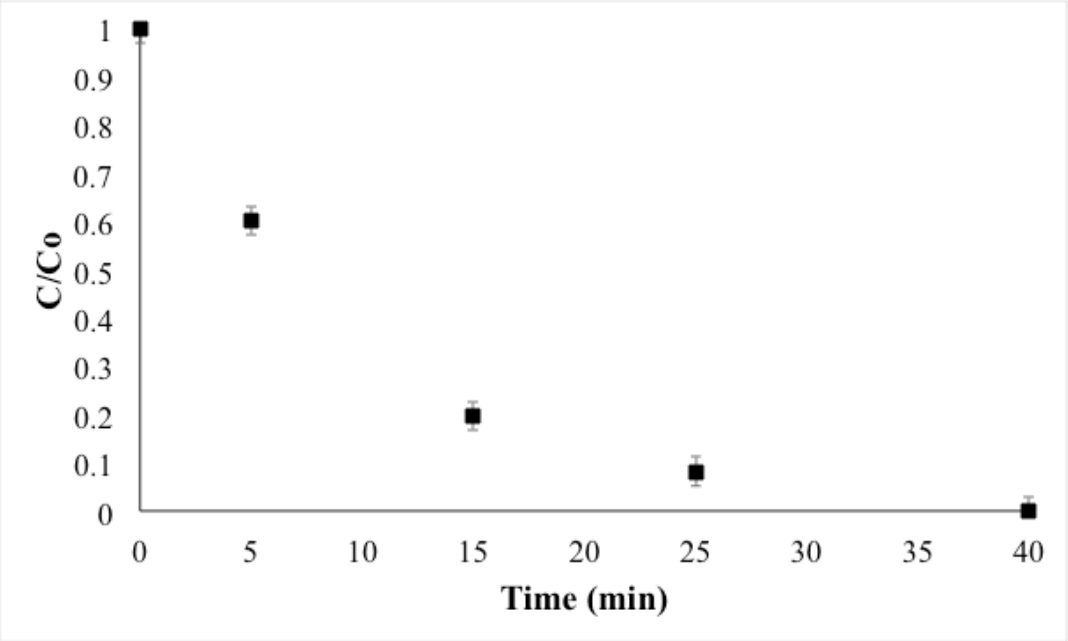

Figure 7.2. Total ammonium nitrogen (TAN) evolution during hollow-fibre membrane contactor tests. C is the TAN concentration and C_O is the initial TAN concentration.

7.3.3. Composting performance

The composting process of ACoD sludge and conventional sludge was evaluated for a maximum of 44 days in 7 reactors. The effect of applying different aeration mode, adding inoculum to the mixtures and mixing sludge with BA in different proportions was assessed. To determine whether the composting process and stabilisation were achieved, mixtures of each reactor were characterised at the beginning (Initial characterisation) and at the end of process (Final characterisation) (Table 7.5).

The initial characterisation showed that C/N ratio was, in general, lower in mixtures generated with volumetric proportions since there was a higher content of sludge in those mixtures. TN content was higher in reactors containing ACoD sludge (R_{A1}, R_{A2} and R_{A3}) than in their replicates containing conventional sludge (R_{C1}, R_{C2} and R_{C3}), due to the presence of microalgae biomass in the ACoD process, which has a high nitrogen content (Ullah et al., 2015). Initial moisture content was around 60% in all the reactors, which is an optimum value to start the composting process (Bueno et al., 2008; Diaz and Savage, 2007). Reactors containing ACoD sludge had higher moisture content associated with the dewatering method used for each sludge. It is also remarkable that in the mixtures generated with volumetric proportions, moisture content was higher than in the ones

generated by theoretical calculations due to their higher proportion of sludge in comparison with the latter (Table 7.5), except in reactor R_{A1}. Initial porosity was in general higher in mixtures with theoretical proportions of sludge and BA. Initial pH and electrical conductivity of both types of mixture had typical initial values according to their ACoD and conventional sludge composition. The initial characterisation of ACoD sludge (not mixed with BA or inoculum) showed no presence of *E. coli* either *Salmonella* spp., microorganisms commonly used as pathogens indicators. Conventional sludge (not mixed with BA or inoculum) contained no *Salmonella* spp. but was positive for *E. coli*. The inoculum added to some reactors () showed the presence of *E. coli*.

Final characterisation of samples (Table 7.5) showed that moisture content in general did not significantly change from the initial conditions. However, reactor R_{C1} lost a significant amount of moisture, from 59% to 48%, associated with an excess of forced aeration that contributed to the dryness of the material. On the other hand, reactor R_{C3} had increased moisture content, from 50% to 66%. This could be explained by inadequate drainage of the excess moisture. pH values were around 8, which are in agreement with typical values of mature compost (8.0 – 8.5 (Diaz and Savage, 2007)). Conductivity values showed an increase in comparison to initial values in all reactors (Table 7.5). According to Diaz and Savage (2007) this behaviour is typical in a composting process due to the mineralisation of the organic matter. All the samples from the reactors (except R_{A2} and R_{C2}) contained *E. coli* after composting process. Since the initial samples from the ACoD reactors were *E. coli* free (Table 7.5), and the initial characterisation of the inoculum showed *E. coli* to be present, it is possible that they were contaminated by the inoculum. Only reactors R_{A2} and R_{C2} achieved complete sanitation after the composting process, showing a final material without *E. coli* and *Salmonella* spp.

For assuring correct performance of all phases occurring during composting process, room temperature and reactors temperature were daily measured (Figure 7.3). Reactors R_{A2} and R_{C2}, with volumetric mixtures, showed temperatures between 50 ºC and 53 ºC achieving the thermophilic temperature necessary for the sanitation of the composted material (Insam and De Bertoldi, 2007). In the case of reactor R_{A2}, the thermophilic phase was reached during a period of 20 days and in reactor R_{C2} during a period of 24 days (Figure 7.3). Reactor R_{A2}, which contained ACoD sludge, had a larger acclimatisation period since it followed the same evolution as reactor R_{C2} (with conventional sludge) but

with a delay of several days (Figure 7.3). This could be associated with a lower content of easily biodegradable organic matter due to the high SRT (70 d) of the previous ACoD process. In both reactors, upper organisms such as mites were found as bioindicators of the correct progress of the composting process (Soliva, 2001). Indeed, these two reactors also showed no presence of *E. coli* and *Salmonella* spp. as mentioned in the paragraph above.

The remaining reactors did not achieve thermophilic temperatures probably due to: the excess of aeration in reactors with forced aeration (R_{A1}, R_{C1}) (Bueno et al., 2008; Negro et al., 2000) coupled with the small volumes of the reactors, which led to higher heating losses; the absence of inoculum in the reactor R_{A4} (Manu et al., 2017); and/or the lower sludge content in the reactors prepared with theoretical proportions (R_{A1}, R_{C1}, R_{A3}, R_{C3}).

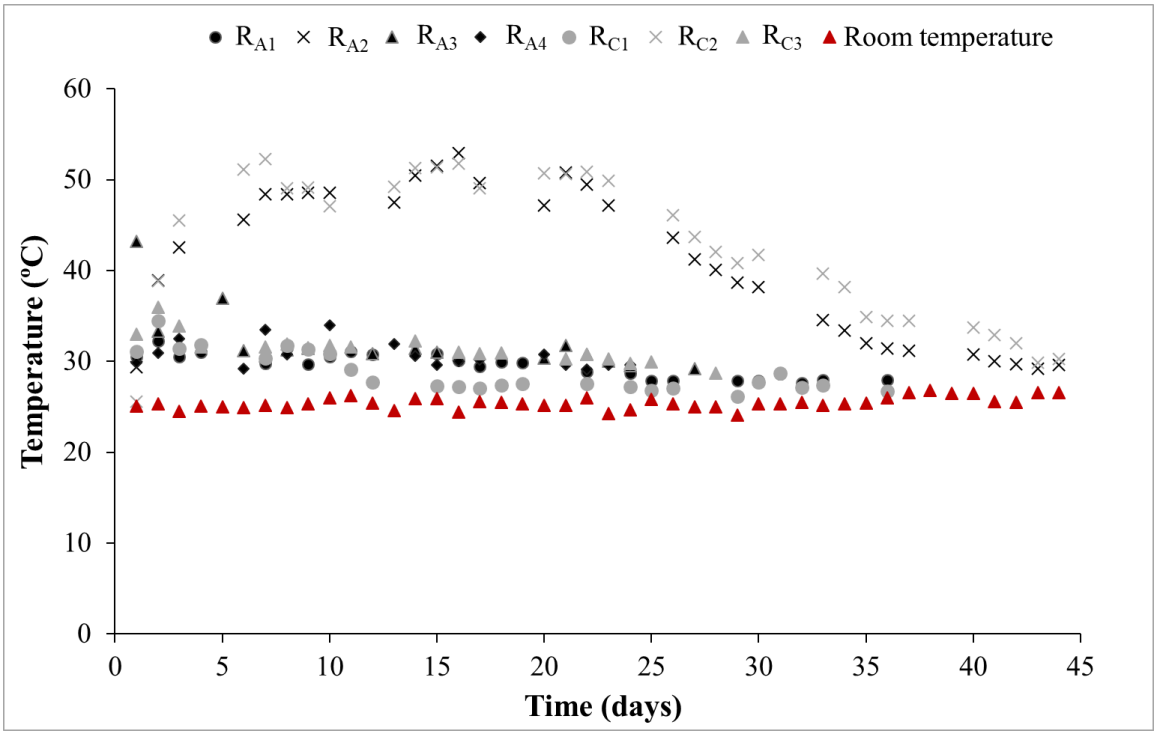

Figure 7.3. Evolution of mixture´s temperature and room temperature during composting period.

Table 7.5. Initial and final characterisation of the composting samples for the seven reactors evaluated.

	ACoD sludge				*Conventional sludge*		
	R_{A1}	**R_{A2}**	**R_{A3}**	**R_{A4}**	**R_{C1}**	**R_{C2}**	**R_{C3}**
Mixtures	Theoretical	v/v	Theoretical	v/v	Theoretical	v/v	Theoretical
Aeration / Inoculum	Forced / Yes	Turned / Yes	Turned / Yes	Turned / No	Forced / Yes	Turned / Yes	Turned /Yes
Initial characterisation							
C/N ratio	24.0 ± 0.7	14.5 ± 0.1	15.8 ± 0.5	16.4 ± 0.1	24.6 ± 0.1	12.5 ± 0.1	19.1 ± 0.1
Moisture content (%)	68.1 ± 0.8	68.7 ± 0.4	59.5 ± 0.9	62.9 ± 0.4	58.6 ± 0.5	64.3 ± 0.7	50.4 ± 0.5
O.M (%)	87.2 ± 0.6	84.1 ± 0.5	75.8 ± 0.9	87.0 ± 0.6	80.1 ± 0.8	86.9 ± 0.5	75.9 ± 0.5
Porosity (%)	36.5 ± 0 .9	13.4 ± 0.9	38.4 ± 0.6	21.6 ±1.5	33.4 ± 2.0	24.0 ± 1.4	40 ± 1.0
TN ($gN \cdot kg_{d.m}^{-1}$)	15.0 ± 1.2	24.6 ± 1.1	15.1 ±1.3	9.6 ± 0.7	11.6 ± 0.9	11.5 ± 0.8	9.1 ± 0.6
pH	7.8 ± 0.1	7.4 ± 0.1	8.1 ± 0.6	6.3 ± 0.1	8.5 ± 0.0	8.5 ± 0.1	8.5 ± 0.1
Conductivity ($\mu S \cdot cm^{-1}$)	1104 ± 158	1418 ± 272	1775 ± 144	2620 ± 190	1905 ± 247	1490 ± 258	1992 ± 249
*E.coli**	A	A	A	A	P	P	P
Salmonella spp.*	A	A	A	A	A	A	A
Final characterisation							
C/N ratio	14.9 ± 0.0	13.0 ± 0.2	14.9 ± 0.0	13.2 ± 0.3	13.7 ± 0.2	11.4 ± 0.1	17.6 ± 0.2
C/N ratio removal (%)	37.9	10.3	5.4	19.5	44.3	8.8	7.8
Moisture content (%)	68.3 ± 0.3	70.0 ± 1.0	61.6 ± 0.5	67.96 ± 0.6	47.8 ± 0 .2	67.8 ± 0.5	66.5 ± 0.4
O.M (%)	76.6 ± 0.2	75.8 ± 0.7	74.8 ± 0.3	82.51 ± 0.5	74.8 ± 0.5	66.8 ± 0.9	72.5 ± 0.3
O.M. removal (%)	12.1	9.8	1.3	5.2	6.5	23.1	4.6
pH	8.3 ± 0.1	8.7 ± 0.1	8.3 ± 0.1	7.8 ± 0.2	8.0 ± 0.1	8.2 ± 0.2	8.11 ± 0.05
Conductivity ($\mu S \cdot cm^{-1}$)	1908 ± 196	2300 ± 253	1990 ± 181	3010 ± 173	2170 ± 260	3530 ± 224	2800 ± 250
E.coli	P	A	P	P	P	A	P
Salmonella spp.	A	A	A	A	A	A	A

O.M: organic matter; TN: total nitrogen; d.m.: dry matter; v/v: volumetric proportions; A: absence of microorganisms in sludge; P: presence of microorganisms in sludge; ´´ pathogens in initial samples were only measured in the sludge, not in the mixtures of sludge and bulking agent.*

The effect of applying different aeration mode was studied in pairs of reactors R_{A1} and R_{A3} and R_{C1} and R_{C3} (Table 7.5). The results indicated that forced aeration offers better composting condition since a higher organic matter and C/N ratio removal was observed in reactors R_{A1} and R_{C1}, compared with their replicates R_{A3} and R_{C3}, which were aerated turning by hand (Table 7.5).

The effect of adding an inoculum source to the sludge-BA mixtures was evaluated in pair of reactors R_{A2} and R_{A4}. The results indicated that inoculum has an important effect on composting process since thermophilic temperature and sanitation was achieved in reactor R_{A2} but not in reactor R_{A4}. Higher organic matter removal (9.8%) was also observed in reactor R_{A2} than in R_{A4} (5.2%).

Finally, the effect of mixing sludge and BA in different proportions (volumetric or theoretical proportions) was evaluated in pairs of reactors R_{A2} and R_{A3} and R_{C2} and R_{C3}. The results indicated that reactors R_{A2} and R_{C2}, which were prepared with volumetric proportions of BA and ACoD and conventional sludge, respectively, achieved thermophilic temperature and showed a complete sanitation of the compost, suggesting that this mixture method should be used for a compost process. Both these reactors were aerated turning by hand and achieved percentages of organic matter removal and C/N ratio removal of 9.8 and 10.3 for reactor R_{A2} and 23.1 and 8.8 for R_{C2}, respectively. In contrast, reactors R_{A3} and R_{C3}, run under the same operating conditions but mixed with theoretical proportions did not achieve thermophilic temperature and showed percentages of organic matter removal and C/N ratio removal of 1.3 and 5.4 for reactor R_{A3} and 4.6 and 7.8 for R_{C3}, respectively, being these percentages lower than the ones observed for the pair of reactors R_{A2} and R_{C2} (Table 7.5).

When comparing all the reactors, of those fed with conventional sludge, R_{C2} showed the best performance in terms of compost sanitation, temperature achieved and organic matter removal efficiency, but not in terms of C/N ratio removal (Table 7.5). Reactor R_{C1} showed the best C/N ratio removal (44%), but did not achieve thermophilic temperature or compost sanitation. Of the reactors fed with ACoD sludge, even reactor R_{A2} showed the best performance in terms of compost sanitation and temperature achieved, organic matter and C/N ratio removal percentages were not the highest values observed in all reactors. Indeed, reactor R_{A1} showed an organic matter and C/N ratio removal percentage of 12.1

and 37.9, respectively, this being the highest value for these two parameters in those reactors fed with ACoD sludge (Table 7.5). The two reactors mentioned (R_{A1} and R_{C1}) were prepared with mixtures calculated by theoretical proportions and forced aeration was applied. The previous results suggest that, although reactors R_{A2} and R_{C2} were the only ones that achieved compost sanitation, and the volumetric proportions seemed to be more suitable for a composting process, a higher organic matter and C/N ratio removal could be achieved if forced aeration would be applied in ACoD sludge reactors, finding the correct airflow to achieve the best composting conditions.

In general, it can be observed that the higher the initial C/N ratio, the greater the elimination of this parameter, which is the case of mixtures generated with theoretical proportions. However, in mixtures generated with volumetric proportions, the amount of sludge added to the mixtures is 3.2 times higher than in the theoretical ones. Therefore, it actually contains more biodegradable organic matter but it also has a much higher N content, which makes the initial C/N ratio lower. In addition, ammonia may have been lost due to the combination of ammonification of the organic nitrogen and the basic pH values reached during the composting process. The latter may result in a not significant change of the initial values of C/N ratio for mixtures generated with volumetric proportions and, thus, in lower C/N ratio removal. Therefore, since C/N ratio is a chemical composting parameter and not a biochemical one, it could be assumed that this alone is not a suitable indicator of the process evolution and that all the parameters (aeration, temperature, moisture content, presence of inoculum, proportions of sludge and BA) should be controlled. New BA and sludge ratios should thus be applied in future experiments to achieve correct C/N ratio removal and material sanitation. The optimum aeration rate flow should also be studied to obtain the optimum composting results.

7.3.3.1. Respirometry: monitoring the biological activity

Biological activity was only measured in the reactors that reached thermophilic temperatures, since this is an indicator of correct process evolution and higher biological activity in the mixture. Respirometric tests were therefore carried out in reactors R_{C2} (conventional sludge) and R_{A2} (ACoD sludge). The RIs at process temperature and 37 ºC are shown in Table 7.6 for each respirometric test performed.

Table 7.6. Respirometric index (RI) obtained in respirometric tests.

Respirometric test	Day of composting	RI ($mgO_2 \cdot gO.M.^{-1} \cdot h^{-1}$)
RI_{A2} (48ºC)	7	3.28
RI_{A2} (52ºC)	15	1.81
RI_{A2} (44ºC)	26	1.51
RI_{C2} (51ºC)	6	4.46
RI_{C2} (52ºC)	16	2.04
RI_{C2} (44ºC)	27	1.72
RI_{C2} (37ºC)	44	0.242
RI_{A2} (37ºC)	44	0.257

O.M.: organic matter

Figure 7.4a, 7.4b and 7.4c show the biological activity measurement for reactor R_{A2} at Days 7, 15 and 26 of operation, corresponding to Days 2, 10 and 21 at the beginning of the thermophilic phase, respectively. The temperature of the process at the moment of the respirometric tests was 48 ºC, 52 ºC and 44 ºC, respectively. Figure 7.4d, 7.4e and 7.4f show the measurement of the biological activity for reactor R_{C2} at Days 6, 16 and 27 of operation, corresponding to Days 4, 14 and 25 at the beginning of the thermophilic phase, respectively. The temperature of the process at the moment of the respirometric tests was 51 ºC on Day 6, 52 ºC on Day 16, and 44 ºC on Day 27.

The slope of the curves marked in red colour in Figure 7.4 represents the oxygen uptake rate. The higher the composting time, the less the slope of the curve. RI thus decreased as composting time increased; so that biological activity also decreased, the first few days of the thermophilic phase being the period with the highest biological activity. The same behaviour than in reactor R_{A2} was observed in reactor R_{C2}. The difference between both reactors is that reactor containing ACoD sludge (R_{A2}) presented lower RI than reactor R_{C2} at the same temperature (Table 7.6). This could be explained by the higher SRT (70 d) in the previous ACoD process than in the conventional AD (SRT of 20 d), which led to a lower biodegradable substrate concentration available for microorganisms, therefore lower biological activity. The thermophilic phase was also longer in reactor R_{C2} than in reactor R_{A2} (Figure 7.3), which also indicates higher biological activity in the mixture.

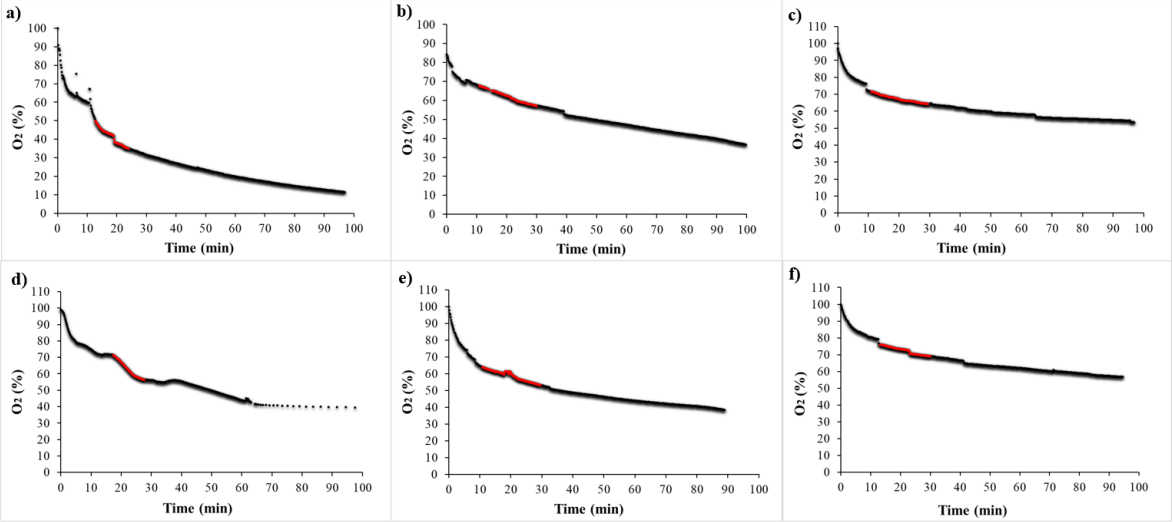

Figure 7.4. Oxygen percentage evolution over time at process temperature in reactors R_{A2} (a, b, c) and R_{C2} (d, e, f).

The respirometric tests at 37 ºC showed that the lower the RI, the more stable the mixture. At the end of the composting process, the biological activity decreased significantly in both reactors, showing similar RI values (Table 7.6). As established by the TMECC (US Department of Agriculture and Council, 2001), a composted material becomes stabilised when RI at 37 ºC is between 0.5-1.5 $mgO_2 \cdot g$ organic $matter^{-1} \cdot h^{-1}$ and it is very stable when RI is below 0.5 $mgO_2 \cdot g$ organic $matter^{-1} \cdot h^{-1}$, such is the case. In terms of stability, the mixtures in reactors R_{C2} and R_{A2} were therefore stabilised (Table 7.6).

Composting process after a previous AD step was thus achieved from both ACoD and conventional sludge. Reactors R_{A2} and R_{C2}, prepared with volumetric proportions achieved thermophilic temperatures and complete compost sanitation. Reactor R_{C2} had higher organic matter removal (2.4-fold higher) but lower C/N ratio removal (1.1-fold lower) than R_{A2}. Both reactors showed RI values associated with a stabilised composted material and the respirometric tests indicated that RI at process temperature diminished when composting time increased. So, even ACoD sludge could present non-biodegradable material associated with the substrates fed to the reactor (microalgae and primary sludge) a composting process was applied, achieving similar values than the ones observed for a conventional sludge composting process.

7.4. CONCLUSIONS

Three potentially useful by-products were generated through microalgae and primary sludge co-digestion in an AnMBR: methane-rich biogas, nitrogen-rich permeate and nutrient-rich digestate. Nitrogen was recovered from the permeate with an efficiency of 99%. An ammonia sulphate solution, which could be used as a commercial fertiliser, was obtained. For the first time, composting process applied to a digestate coming from a microalgae co-digestion plant was evaluated in the present work at laboratory-scale. ACoD digestate composting was compared to a conventional AD digestate composting, and similar values were obtained for both. The best composting performance in terms of sanitation of the composted material and removal of organic matter and C/N ratio was observed when mixing sludge with BA in volumetric proportions (2.5 volume of BA per 1 volume of sludge), applying forced aeration and adding an inoculum from an industrial compost plant to accelerate the biological process. Respirometric tests indicated a highly stable final composted material. The combination of a microalgae co-digestion system with subsequent composting process offers complete resource recovery (energy, nutrients and water) from sewage in a circular economy-based scenario for future WRRF implementation.

CRediT authorship contribution statement

Rebecca Serna-García: Conceptualization, Methodology, Investigation, Formal analysis, Writing - Original draft. Patricia Ruiz-Barriga: Methodology, Writing – Original draft. Guillermo Noriega-Hevia: Formal analysis, Writing – Original draft. Joaquín Serralta: Writing – Review & Editing. María Pachés: Supervision; Writing – Review & Editing. Alberto Bouzas: Supervision, Writing – Review & Editing, Funding acquisition.

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Overall discussion

8. OVERALL DISCUSSION

Microalgae cultivation has been thoroughly studied during the last decades in the field of WT. Consequently, AD of wastewater grown microalgae is receiving increased attention, but there are still some problems which hinder a high efficiency of the process, such as the high resistance to biodegradation that their cell walls present or the low C/N ratio that microalgae biomass presents. Co-digestion with carbon-rich wastes is a strategy that could overcome these disadvantages, offering a high stable system and avoiding a possible inhibition by free ammonia. The initial hypothesis of this thesis stated that ACoD of microalgae with primary sludge could improve microalgae AD performance, producing more biogas due to the better degradation of the co-substrate. Following the previous hypothesis, it was thought that microbial community involved in ACoD process would change compared to that observed during microalgae AD.

ACoD of microalgae with sewage sludge has been studied previously in batch or short-term experiments at mesophilic conditions. However, scarce studies have focused on a long-term continuous system and none of the studies have been carried out working at thermophilic conditions. The aim of the present thesis is the long-term evaluation of a continuous ACoD system treating raw microalgae coming from a MPBR plant treating wastewater and primary sludge coming from a WWTP plant. This thesis studies ACoD process both at mesophilic and thermophilic conditions. This work also offers the assessment of the microbial community involved in the co-digestion process at different temperatures. The ACoD process was scaled-up from laboratory to pilot-scale and studied for a year. Filtration process was also evaluated at pilot-scale and potential resource recovery was assessed.

8.1. ANAEROBIC CO-DIGESTION EXPERIMENTS: EFFECT OF DIFFERENT OPERATING CONDITIONS ON BIOLOGICAL PROCESS.

ACoD of microalgae and primary sludge was studied both at laboratory and pilot-scale. First experiments were carried out at laboratory-scale in order to find the best operating conditions to scale-up the process. 9 L CSTRs coupled to an ultrafiltration membrane

tank were used for ACoD experiments at lab-scale. Different SRT, temperature and OLR were studied at lab-scale.

8.1.1. Solids retention time effect

Two periods (100 d SRT and 70 d SRT) were studied in a mesophilic AnMBR that was operated for 830 d at lab-scale. During these two periods, HRT and OLR were kept constant at 30 d and 0.5 $gCOD \cdot L^{-1} \cdot d^{-1}$, respectively. High process stability in terms of alkalinity, VFA production and pH was observed with both operating conditions. However, higher methane production efficiency was observed working at 100 d SRT. Methane yields of 256 $mLCH_4 \cdot gCOD_{inf}^{-1}$ (435 $mLCH_4 \cdot gSV_{inf}^{-1}$) and 225 $mLCH_4 \cdot gCOD_{inf}^{-1}$ (391 $mLCH_4 \cdot gTVS_{inf}^{-1}$) were observed at 100 and 70 d SRT, respectively, which corresponds to biodegradability percentages of 73.1 and 64.6%. Then, 14% higher methane production can be achieved working with 30 d higher SRT, since a higher hydrolytic and methanogenic activity is promoted with higher SRT. These results are in accordance with Greses (2017) and Giménez et al. (2017), who also observed that the higher the SRT, the higher the methane yield, working in an AnMBR with microalgae as only substrate.

The effect of doubling the SRT was also studied with a second reactor connected to the mesophilic AnMBR during period PI (when reactor was operated at 100 d SRT). This second reactor was a CSTR, named as AnR, that was fed with the daily AnMBR purge, and, as it was not connected to a membrane, HRT and SRT were set at 100 d. This reactor was used as a biomass reservoir but biological process evolution was also monitored. This second mesophilic reactor showed a methane yield of 35 $mLCH_4 \cdot gCOD_{inf}^{-1}$ which corresponds to an average biodegradability of 10.2%. Hence, the second reactor enabled a 5 percentage point increase in the total methane production offering a final 78% biodegradability percentage.

8.1.2. Temperature effect

Two temperatures, 35 ºC and 55 ºC, were tested in a laboratory AnMBR. The increase in temperature, keeping constant the rest of operating conditions, allowed a rise in methane yield of 8% (from 225 $mLCH_4 \cdot gCOD_{inf}^{-1}$ (391 $mLCH_4 \cdot gTVS_{inf}^{-1}$) to 242 $mLCH_4 \cdot gCOD_{inf}^{-1}$ (429 $mLCH_4 \cdot gSV_{inf}^{-1}$)). Greses et al. (2018) also observed a

higher methane production when increasing operating temperature for microalgae digestion. Temperature conditions affect the development of digester's microbial community (Kougias and Angelidaki, 2018) and a higher temperature is reported to increase hydrolytic activity of the system, achieving a higher long fatty acids degradation (Labatut et al., 2014).

8.1.3. Membrane implementation effect

A thermophilic lab-scale CSTR was started-up for microalgae and primary sludge ACoD with an OLR of 0.22 $gCOD \cdot L^{-1} \cdot d^{-1}$. VFA accumulation (1480 $mgCH_3COOH \cdot L^{-1}$) and NH_3 accumulation (29.9 $mgNH_3\text{-}N \cdot L^{-1}$) was observed after a few days of operation. The alkalinity observed was very low (454 $mgCaCO_3 \cdot L^{-1}$), showing the system a VFA alkalinity / total alkalinity ratio of 0.7, value that was above the maximum recommended (0.4) for a stabilised digester. Then, due to the VFA and NH_3 accumulation observed, joint to the fact that biogas was not being produced, it can be assumed that the system was inhibited. Then, a ceramic membrane was installed to allow operating at a lower HRT (30 d) without changing the SRT (70 d). The reactor became an AnMBR and, after 130 d of operation, pseudo-steady state was achieved, biodegradability reached a percentage of 51.4% and no sign of inhibition was observed. The microbial community characterisation also showed a change with membrane implementation: most of the groups observed before membrane installation were not present after the installation, as will be further discussed in Section 8.5.2. Then, the AnMBR configuration improved the performance of the thermophilic ACoD, allowing the system operation at a higher OLR without observing FAN or VFA inhibition.

8.1.4. Organic loading rate effect

A progressive increase in OLR from 0.17 to 0.5 $gCOD \cdot L^{-1} \cdot d^{-1}$ was studied in a thermophilic lab-scale AnMBR operated for 700 d, divided in four experimental periods. The increase in OLR led to an increase in methane production. Indeed, a positive Pearson's correlation (0.828) between methane yield and OLR was found when applying a bivariate Pearson correlation analysis. The threefold OLR rise improved methane yield by 35%. This improvement in methane yield could be due to a higher hydrolytic activity that allows also a higher methanogenic activity. In fact, higher VFA production was observed with higher OLR. The higher VFA production led to a temporary VFA

accumulation, at the beginning of periods PI and PII, but the system stability was quickly recovered. NH_3 concentration was also increased at the beginning of each period due to the higher NH_4 release during AD process, but no inhibition of the system was observed. Indeed, the highest alkalinity concentration (2030 $mgCaCO_3 \cdot L^{-1}$) was found working at 0.5 $gCOD \cdot L^{-1} \cdot d^{-1}$. This fact demonstrates the robustness of the system, that shows no inhibition by overloading.

8.1.5. Operating conditions chosen for the scale-up

The AnMBR configuration was chosen for scaling-up the co-digestion process. Regarding SRT, as an increase in biodegradability from 65 to 73% was observed at lab-scale when increasing SRT from 70 to 100 d, an economic balance was carried out to know which is the best condition to operate a pilot plant. The economic balance indicated 3% higher annual benefits when operating a 70 d SRT system. Moreover, the payback period for a 100 d SRT system would be 15.8 years and 10.7 years for a 70 d SRT system. Regarding temperature, as an increase in biodegradability from 65 to 69% was observed at lab-scale when rising temperature from 35 to 55 ºC, an energy balance was carried out to evaluate which is the best operating condition. Energy balance calculations showed a 5.5-fold increase in energy demand working at thermophilic temperature comparing to mesophilic. Thus, 70 d SRT and 35 ºC were chosen to operate the ACoD pilot plant. The AnMBR pilot plant was operated at the highest OLR studied at lab-scale: 0.5 $gCOD \cdot L^{-1} \cdot d^{-1}$.

8.2. ANAEROBIC CO-DIGESTION PILOT PLANT

The ACoD pilot plant was operated for a year outdoors. The system showed a good performance, achieving high efficiencies in terms of methane production and organics removal. The primary sludge and microalgae degradation led to a biogas production of 78 $Lbiogas \cdot d^{-1}$, which corresponds to 154 $mLbiogas \cdot d^{-1} \cdot L^{-1}_{reactor}$. Then, the anaerobic co-digester achieved a methane yield of 215 $mLCH_4 \cdot gCOD_{inf}^{-1}$ (370 $mLCH_4 \cdot gTVS_{inf}^{-1}$) and 64% efficiency in VS removal. The system also showed a high stability since all VFA produced were being converted to methane and no VFA accumulation was observed. Indeed, a very low VFA alkalinity / total alkalinity ratio was found, indicating a well-stabilised anaerobic process. Although some operational problems were found when operating the ACoD pilot plant, related with gas leakages or equipment, these problems

were solved and were useful to indicate which are the weak aspects to control for operating a future large-scale plant. Overall, the results obtained at pilot-scale were in accordance with those obtained at lab-scale working at the same operating conditions, which is a sign of the reproducibility of the system, making the future industrial scale-up promising.

8.3. COMPARISON WITH MICROALGAE MONO-DIGESTION

The values of biodegradability percentages and methane yields observed in the ACoD experiments are higher than those observed for microalgae mono-digestion under different configurations. Greses (2017) studied microalgae (*Chlorella* and *Scenedesmus*) AD under the same configuration (CSTR and AnMBR) used in the present work, so, the ACoD system could be compared with a microalgae mono-digestion system. Greses (2017) reported a methane yield of 148.5 $mLCH_4 \cdot gCOD_{inf}^{-1}$ when digesting *Scenedesmus* microalgae in a mesophilic AnMBR operated at an SRT of 70 days. That methane yield is lower than the value observed in the present work (225 $mLCH_4 \cdot gCOD_{inf}^{-1}$), operating at the same conditions. This author also reported 164 $mLCH_4 \cdot gCOD_{inf}^{-1}$ of methane yield when digesting *Scenedesmus* spp. at 0.3 $gCOD \cdot L^{-1} \cdot d^{-1}$ of OLR and 70 d SRT in a thermophilic CSTR. In the present work, with the same operating conditions (temperature, OLR and SRT) a methane yield of 229 $mLCH_4 \cdot gCOD_{inf}^{-1}$ was obtained. Other works on microalgae mono-digestion also showed lower methane production. For instance, González-Fernández et al. (2013) observed a biodegradability of 9.4% (33 $mLCH_4 \cdot gCOD_{inf}^{-1}$) digesting untreated *Scenedesmus* spp. in a mesophilic CSTR. Passos et al. (2014) reported 120 $mLCH_4 \cdot gCOD_{inf}^{-1}$ when digesting *Scenedesmus* and *Chlorella* grown in high rate ponds for WT and also observed that methane yield was enhanced by 42% with microwave treatment (170 $mLCH_4 \cdot g\ COD_{inf}^{-1}$). Methane yields obtained in the present work are also higher than those obtained digesting pretreated microalgae (Magdalena et al., 2018, Passos et al., 2014). Moreover, previous experiments carried out in batch reactors showed that microalgae and primary sludge mono-digestion yielded 62% and 64% biodegradability (Martínez, 2018), respectively, working at 100 d SRT. The biodegradability obtained in the present work for microalgae and primary sludge ACoD at 100 d SRT was 73%. Then, microalgae and primary sludge ACoD can substantially improve microalgae mono-digestion and primary sludge mono-digestion and boost methane production without applying costly pretreatments.

This enhancement in methane production could theoretically be explained by an increase in the influent C/N ratio due to the addition of primary sludge to microalgae substrate. However, C/N ratios of around 5 and 9 for microalgae and primary sludge, respectively, were found in the substrates used in this work. So, these values were apparently not the optimal ones for AD (Yen and Brune, 2007). Nevertheless, high biodegradability and methane yield were achieved in the present work as reported in the previous sections. Primary sludge is an easily biodegradable substrate since is mainly formed by colloidal organic matter and, co-digesting it with microalgae seems to have synergy positive effects on substrate degradation, as will also be confirmed by the microbial analysis (Section 8.5) that shows the syntrophic network created for ACoD process.

8.4. FILTRATION EXPERIMENTS

Filtration experiments were carried out in both laboratory and pilot-scale mesophilic reactors. Results at lab-scale indicated that the system was stable during the 30 d the experimentation lasted, working at an average J_{20} of 5.8 LMH, keeping membrane permeability values around 35 LMH·bar^{-1}. This membrane permeability value was low, possibly due to the mixed liquor viscosity (around 1.5 cP) but it was kept stable during 30 d, suggesting that no irreversible fouling was expected at long-term operation. The short-term flux analysis evaluation showed similar filtration resistances during the experiment even at relatively high fluxes (7-10.5 LMH), which corroborated the hypothesis about not expecting irreversible fouling problems. The SMPs concentration in the reactor also remained stable during the experiment. Despite the high lipids content presented by microalgae and primary sludge, the proteins and carbohydrates were found at concentrations around 15 and 54 mg·L^{-1}, respectively, so no SMPs accumulation that hinders filtration process was observed.

Filtration experiments at pilot-scale were carried out to find the optimal membrane operation and to know the membrane behaviour at long-term operation. Results from the four experiments with different SGD, filtration time and J_{20} conditions indicated that J_{20} of 4.2 LMH, filtration time of 160 s and SGD of 0.15 N·m^3·m^{-2}·h^{-1} seemed to be the most appropriate conditions for operating a microalgae and primary sludge ACoD pilot plant filtration process.

8.5. MICROBIAL CHARACTERISATION

Microbial community involved in the ACoD process was studied in the two lab-scale mesophilics reactors and in the lab-scale thermophilic reactor. In the three reactors, microbial community was mainly composed of bacteria, since this group is involved in three of the four main stages of the AD process and has a wide variety of substrate preferences, but archaea were also present, being the responsible for the production of biogas riched in methane. A similar microbial core in which polysaccharide and protein-degrading bacteria and syntrophic microorganisms were predominant was found in mesophilic and thermophilic reactors, since the same substrates were being degraded.

8.5.1. Mesophilic reactors

Mesophilic AnMBR and AnR samples as well as inoculum samples were analysed. Chloroflexi, Proteobacteria, Caldiserica, Firmicutes, Cloacimonetes, Bacteroidetes, Thermotogae and Synergistetes were the predominant phyla found in the mesophilic reactors, Proteobacteria and Chloroflexi being the most abundant ones. Microalgae and primary sludge co-digestion led to the dominance of Anaerolineaceae members (phylum Chloroflexi), which are saccharolytic bacteria. This group was already present in the inoculum sample (coming from a reactor digesting microalgae), but its relative abundance decreased in the reactor, suggesting that this group was involved in microalgae degradation, as reported previously by Gonzalez-Fernandez et al. (2018) and Greses et al. (2018). Other hydrolytic microorganisms such as members of Thermotogae (*Mesotoga*, *Defluviitoga*) and Lentimicrobiaceae, involved in polysaccharides degradation, were also found. Proteolytic microorganisms from phyla Synergistetes and Firmicutes also had an important role in microalgae and primary sludge degradation. Syntrophic microorganisms had a remarkable presence in the co-digester. For instance, *Smithella*, a bacterial group involved in the conversion of propionate into acetate, was present in a high abundance in the AnMBR. This microorganism has been described as a common syntrophic partner of *Methanosaeta*, a group of archaea present in the co-digester. The presence of these two groups can thus explain the production of a methane-rich biogas. Other syntrophic microorganisms such as Rikenecellaceae members (Bacteroidetes phylum), which are involved in amino acids degradation in syntrophic association with hydrogenotrophic methanogens, were also found.

In general, mesophilics AnMBR and AnR had in common the majority of groups involved in ACoD, although the relative abundances percentages were different. Nevertheless, the beta diversity analysis through Simpson Evenness Index indicated that diversity was higher in the AnMBR than AnR samples, with a higher number of species, probably due to the presence of an ultrafiltration membrane in the AnMBR, which has been reported to increase microbial community diversity in terms of species richness (Greses et al., 2018). The beta diversity analysis also showed a shift from inoculum to AnMBR samples. Some groups related to microalgae degradation stayed in the reactor but the presence of a new substrate (primary sludge) changed the microbial core and created new relations between microorganisms to assure the methane production through the substrates degradation.

8.5.2. Thermophilic reactor

Microbial community was characterised during the start-up, before membrane installation (the thermophilic reactor was a CSTR), as well as during the four periods investigated in the thermophilic AnMBR. Coprothermobacteraota, Thermotogae, Hydrothermae, and Proteobacteria, followed by Firmicutes, Bacteroidetes, Actinobacteria and Synergistetes were the predominant phyla. *Coprothermobacter*, involved in protein disruption, was one of the most abundant genera in the AnMBR. Another proteolytic microorganism from phylum Synergistetes (*Acetomicrobium*), which has been described as a syntrophic bacterium (Oosterkamp et al., 2019) was also observed. Hydrolytic microorganisms involved in polysaccharides degradation were found in the reactor. *Fervidobacterium* and *Defluviitoga* were present in high abundance; *Thermogutta* and *Candidatus Caldatribacterium* were also present in significant abundance. *W5*, a bacterial group involved in propionate degradation (acetate being one of the products of this degradation) had a remarkable presence in the thermophilic AnMBR, especially when OLR was high. *W5* provides acetate to aceticlastic methanogens such as *Methanosaeta* (the most abundant archaea in the co-digester). Nevertheless, the highest abundance of this microorganism matches with the highest abundance of the hydrogenotrophic methanogen *Methanoculleus*, since propionate degradation provides H_2 for hydrogenotrophic methanogenesis.

Beta diversity analysis showed a shift in microbial community after membrane installation. Some species observed before membrane installation such as *Arcobacter* or *Pseudomonas* disappeared after membrane installation and other microorganisms such as *Fervidobacterium* or *Hydrothermae* genus appeared after membrane installation. Regarding the microbial community evolution during the progressive increase in OLR, similar diversity was observed in the four periods, but there was a change in the microorganisms' relative abundance when working at different OLR.

The results presented in Sections 8.5.1 and 8.5.2 suggest the presence of a common microbial core working at both mesophilic or thermophilic temperatures. The marked presence of fermentative, hydrolytic and proteolytic microorganisms is related to the significant content in protein of microalgae and the presence of cellulose in both microalgae and primary sludge. Some hydrolytic microorganisms such as *Coprothermobacter* and *Defluviitoga,* among others, and methanogens such as *Methanosaeta,* were present in both reactors. The remarkable contribution of a syntrophic network with hydrogen-producing bacteria such as *Smithella*, *W5*, *Defluviitoga* or *Acetomicrobium* showed the importance of these groups in microalgae and primary sludge co-digestion.

8.6. RESOURCE RECOVERY

Biogas riched in methane and effluents riched in nutrients were obtained through microalgae and primary sludge ACoD. Biogas had a high methane content (70%) that could be used for energy generation (further research is needed). Nutrients recovery was studied in the permeate and digestate from the ACoD pilot plant. Around 400 $mgNH_4\text{-}N \cdot L^{-1}$ and 37 $mgPO_4\text{-}P \cdot L^{-1}$ were released from ACoD process. The ammonium content in the effluent was higher than that present in the influent stream, but in the case of phosphate, the concentration was lower than that in the influent. This result suggests a possible P precipitation taking place in the reactor. For that reason, a P balance was carried out and results indicated a P precipitation percentage of 74%. Then, only 26% of the phosphate was available for recovery in the effluent, so, recovery process through struvite precipitation was not studied. Nevertheless, nitrogen recovery was studied through a hydrophobic polypropylene HFMC at laboratory-scale, obtaining a recovery efficiency of 99% in an operation time of approximately 40 min. An ammonia sulphate

solution with a maximum N richness of 4% was obtained. This solution is an inorganic salt that could be used as a commercial fertiliser, being a potential substitute for currently used chemical fertilisers.

Composting of the digestate coming from the ACoD pilot plant was also evaluated at lab-scale using Dewar reactors. Results from composting process were compared to results obtained composting a conventional sludge coming from the Carraixet WWTP digester. Different operating conditions related to aeration mode, addition of inoculum and mixing of sludge with BA were tested. Reactors in which force aeration was applied achieved a higher organic matter and C/N ratio removal, compared to reactors turned by hand. Adding an inoculum from an industrial plant seemed to be important for accelerating biological process. Reactors prepared with volumetric proportions (2.5 volume of BA per 1 volume of sludge), achieved thermophilic temperatures and a complete sanitation of the composted material. Both ACoD sludge and conventional sludge were composted obtaining a stable final material. Some differences in RI at process temperature were observed between the two kind of sludges since the ACoD sludge came from a previous AD process operated at a high SRT (70 d) while the conventional sludge came from a previous AD process operated at 20 d SRT and then, the substrates were more degraded in the ACoD pilot plant. Nevertheless, respirometric indexes at 37 ºC were below 0.5 $mgO_2 \cdot g$ organic $matter^{-1} \cdot h^{-1}$, which is an indicator of stability. The final material obtained with both sludges was free of pathogens *E. coli* and *Salmonella* spp., and then, it was sanitised.

8.7. OVERVIEW

Microalgae and primary sludge ACoD was successfully scaled-up from laboratory to pilot-scale and was studied for one continuous year. This is the first step for future industrial scale-up, since it is an indicator of the good reproducibility of the system. Besides, several problems that are not occurring at lab-scale could occur at pilot-scale, working with a much higher volume of reaction, bigger pumps and automation. Finding those problems (e.g. biogas leakage, membrane fouling) helps to know which are the weaknesses of the equipment used or the process *per se* and solve them for future applications.

The synergetic effect occurring when co-digesting microalgae and primary sludge together allowed a higher substrates degradation than that obtained digesting substrates alone, offering high efficiencies in organics removal and methane production. Microbial characterisation results showed that syntrophic relations were occurring between microorganisms resulting in a stable microbial community capable of degrading both substrates. The high stability observed in the system and the high biodegradability was associated not only with the fact that co-digesting both substrates offered synergy effects but also with the fact that implementing a membrane to the system offered advantages. Disruption of *Scenedesmus* or *Chlorella* was achieved without requiring costly pretreatments, which makes ACoD in an AnMBR a promising alternative that saves costs and recycle resources.

Useful resources (methane, nutrients, compost and reclaimed water) were recovered from the ACoD pilot plant. Since the AnMBR allowed obtaining liquid and solid effluents, resources were recovered through three streams: biogas, permeate and digestate. Then, resources are being recovered from wastewater-grown microalgae and primary sludge. The ACoD pilot plant is part of a sustainable scheme in which organics are being removed from raw wastewater through an AD process; nutrients from AD effluent are being removed by microalgae cultivation (offering a liquid effluent that complies with discharge limits); and the final ACoD of waste streams (microalgae and primary sludge) is used for biogas and nutrient-rich by-products production. This scheme encompasses green technologies in a circular economy scenario for WT that is strongly necessary nowadays to decrease the use of resource consuming technologies.

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Conclusions and future perspectives

9. CONCLUSIONS AND FUTURE PERSPECTIVES

9.1. CONCLUSIONS

This thesis has focused on the microalgae and primary sludge ACoD process evaluation at laboratory and pilot-scale, working at different operating conditions. The main aims of the study were: i) to evaluate which are the best operating conditions at lab-scale to scale-up the process, ii) to scale-up the process and study it at long-term, iii) to know which is the microbial community involved in microalgae and primary sludge anaerobic co-digestion and how it is affected by operational changes, iv) to study the filtration process and v) to assess the potential resource recovery. The main conclusions of the work are listed below:

I. 189 mLbiogas·d^{-1}·$L_{reactor}^{-1}$ were produced at SRT of 100 d, HRT of 30 d and OLR of 0.5 gCOD·L^{-1}·d^{-1} in a lab-scale mesophilic AnMBR, yielding higher biodegradability (73%) than that achieved by microalgae (62%) or primary sludge (64%) mono-digestion. The high stability of the system, the absence of VFA or NH_3 inhibition and the high methane yield obtained (256 mLCH_4·$gCOD_{inf}^{-1}$) suggest that a synergism is occurring between both substrates to improve their degradation at high SRT.

II. The decrease in SRT from 100 to 70 d in the mesophilic AnMBR generated 14% lower methane production. 163 mLbiogas·d^{-1}·$L_{reactor}^{-1}$ were produced, which resulted in a biodegradability percentage of 65% and a methane yield of 225 mLCH_4·$gCOD_{inf}^{-1}$.

III. The study of a second lab-scale mesophilic reactor (AnR) connected to the main mesophilic reactor (AnMBR) working at 100 d SRT showed that the main substrates degradation was occurring in the first reactor: 96.4% of the total biogas obtained was being produced in the AnMBR. Nevertheless, the second reactor allowed a 5 percentage point increase in the total methane production while the system achieved an overall 78% biodegradability percentage.

IV. A thermophilic CSTR was started-up with an OLR of 0.22 gCOD·L^{-1}·d^{-1} and SRT and HRT of 70 d, resulting in NH_3 and VFA accumulation of 29.9 mgNH_3-N·L^{-1}

and 1480 mg$CH_3COOH \cdot L^{-1}$, respectively, which is an indicator of AD inhibition. The implementation of a ceramic membrane to the system allowed the correct operation of the reactor. The thermophilic AnMBR, working at the same or higher OLR than the thermophilic CSTR, produced biogas riched in methane and no inhibition by NH_3 was observed.

V. Tripling the OLR (from 0.17 to 0.5 $gCOD \cdot L^{-1} \cdot d^{-1}$) in a thermophilic AnMBR led to a biodegradability increase of 35%, improving system performance and stability.

VI. The thermophilic lab-scale reactor produced 165 $mLbiogas \cdot d^{-1} \cdot L_{reactor}^{-1}$, working at 0.5 $gCOD \cdot L^{-1} \cdot d^{-1}$, 70 d SRT and 30 d HRT, reaching 69% biodegradability and methane yield of 242 $mLCH_4 \cdot gCOD_{inf}^{-1}$, which is a higher yield than the one obtained working at mesophilic conditions.

VII. Economic and energy balances showed 3% higher annual costs when operating a plant at 100 d SRT compared to 70 d SRT and 5.5-fold increase in energy demand when operating at 55 ºC compared to 35 ºC. Then, 70 d SRT and 35 ºC were chosen as the operating conditions to scale-up the process.

VIII. The microalgae and primary sludge ACoD pilot plant was operated continuously during a year, generating 154 $mLbiogas \cdot d^{-1} \cdot L_{reactor}^{-1}$, corresponding to methane yield of 215 $mLCH_4 \cdot gCOD_{inf}^{-1}$. Results obtained at pilot-scale were in accordance with those obtained at lab-scale, demonstrating that lab-scale experiments could be useful to evaluate the effect of different parameters on biological process.

IX. A shift in microalgae species from *Chlorella* to *Scenedesmus* or vice versa was observed during ACoD evaluation and no change in biogas production was noticed, demonstrating the robustness of the system to degrade microalgae at high efficiencies even the predominant specie changed.

X. Membrane permeability was stable during the period (one month) studied at lab-scale and was around 35 $LMH \cdot bar^{-1}$. TMP fluxes were also stable working at different permeate fluxes (3.5, 7.0 and 10.5 LMH). Results from this short-term

experiment suggest the membrane could be operated continuously without expecting irreversible fouling.

XI. Filtration process experiments at pilot-scale indicated that applying SGD (0.15 $N \cdot m^3 \cdot m^{-2} \cdot h^{-1}$), a filtration time of 160 s and conducting a backwash every two filtration cycles was enough for controlling fouling formation.

XII. Microbial community involved in microalgae and primary sludge co-digestion was dominated by hydrolytic microorganisms degrading polysaccharides and proteins, belonging mainly to phyla Chloroflexi, Firmicutes, Thermotogae, Synergistetes, Coprothermobacteraeota and Hydrothermae. Syntrophic relations between microorganisms were key for both substrates degradation: microorganisms such as *Smithella*, *W5* or *Defluviitoga* were involved in the conversion of propionate into acetate, providing acetate to aceticlastic methanogens as *Methanosaeta* and H_2 for hydrogenotrophic methanogens as *Methanoculleus*.

XIII. Beta-diversity analysis through Simpson and Shannon-Wiener estimators and PCoA analysis showed that microbial community evolved from inoculum (coming from a microalgae digester) to ACoD reactor samples. Temperature and membrane implementation to the system also caused a change in microbial population. The increase in OLR studied in the thermophilic lab-scale reactor led to a change in the relative abundance of main microorganisms. Some microorganisms such as *W5* were not present at low OLR.

XIV. The microbial characterisation in the two connected mesophilic reactors indicated a high stability in the system, since the predominant microbial groups in the AnMBR were kept also in the AnR although in lower relative abundances in the AnR. A higher number of species was observed in the AnMBR than in the AnR (3852 vs. 2831 $OTU_{0.97}$), due to the presence of an ultrafiltration membrane.

XV. Nutrient-rich permeate and digestate were obtained from ACoD, allowing nutrients to be recovered from the effluents. Nitrogen was recovered from permeate through a hydrophobic polypropylene HFMC at laboratory-scale, with

an efficiency of 99%. Phosphate was not recovered from permeate since only 26% was available for recovery due to the precipitation processes occurring during AD.

XVI. Lab-scale composting of the digestate allowed obtaining a stable and sanitised (pathogens free) composted material. An inoculum from an industrial composting plant was added to composting reactors to speed up biological reactions. The addition of BA to composting samples was key for composting process: adding 2.5 volume of BA per 1 volume of sludge allowed composting process to be completed.

9.2. FUTURE PERSPECTIVES

Microalgae and primary sludge ACoD has been studied at laboratory and pilot-scale and different operating conditions have been evaluated. However, even results from economic and energy balances have been given to select the more recommendable conditions to operate the pilot plant, these results were based on basic balances. Therefore, a deeper economic analysis and a life cycle assessment has to be considered for future industrial-scale implementation.

The ACoD pilot plant plays a key part in a sustainable scheme for WT. As waste sludge is being produced in the first step of this scheme (in the AD plant for raw WT), the addition of this sludge to microalgae and primary sludge should be also evaluated. In the line of this sustainable scheme, biogas that was being produced during ACoD was rich in methane, but it could be even richer if some techniques based on biogas upgrading were applied.

Regarding nutrients recovery, it has been evaluated for the first time at lab-scale. A correct composting of the samples has been obtained only in two of the seven reactors operated and some parameters such as organic matter removal or C/N ratio removal were not in their maximum values in those reactors so, new operating conditions, based on laboratory results, should be tested at a major scale. Besides, compost obtained could be tested in soil to know if using it as a soil improver is a feasible option.

Appendix

APPENDIX: ABBREVIATIONS

ACoD: Anaerobic co-digestion

AD: Anaerobic digestion

AF: Anaerobic filter

AFBR: Anaerobic fluidized bed reactor

Alk: Alkalinity

AnMBR: Anaerobic membrane bioreactor

AnR: Anaerobic reactor

BA: Bulking agent

BMP: Biochemical methane potential

C_C: Construction costs

CH_4: Methane

CHP: Combined heat and power

CIP: Clean in place

COD: Chemical oxygen demand

CSTR: Continuous-stirred tank reactors

cP: Centipoise

C/N: Carbon/nitrogen

D_C: Depreciation costs

DESASS: Design and simulation of activated sludge systems

DNA: Deoxyribonucleotide

DV: Degasification vessel

EDS: Energy-dispersive X-ray spectroscopy

EGSB: Expanded granular sludge bed

ET: Equalisation tank

FAN: Free ammonia nitrogen

F-B: Filtration-backwash

FISABIO: Fundación para el Fomento de la Investigación Sanitaria y Biomédica de la Comunitat Valenciana

F-R: Filtration-relaxation

FT: Foam trap

f_T: Temperature correction factor

FW: Food waste

GC-FID: Gas chromatograph with a flame ionisation detector

HF: Hollow-fibre

HFMC: Hollow-fibre membrane contactor

HRAS: High rate activated sludge

HRT: Hydraulic retention time

HTS: High-throughput sequencing

ICA: Instrumentation, control and automation

J: Permeate flux

J_{20}: 20 ºC-normalised permeate flux

K: Membrane permeability

K20: 20 ºC-normalised membrane permeability

LMH: $L \cdot m^{-2} \cdot h^{-1}$

MEC: Microbial electrolysis cells

MFC: Microbial fuel cells

MPBR: Membrane photobioreactor pilot plant

MPN: Most probable number

MT: Membrane tank

MWCO: Molecular weight cut-off

N: Nitrogen

NH_3: Ammonia

NO_2: Nitrite

OLR: Organic loading rate

ORP: Oxidation-reduction potential

OTU: Operational taxonomic units

$OTU_{0.97}$: 3% dissimilarity operational taxonomic units

P: Phosphorus

PBR: Photobioreactor

PCoA: Principal coordinates analysis

PCR: Polymerase chain reaction

PE: Population equivalent

POB: Propionate oxidising bacteria

PVC: Polyvinyl chloride

PT: Purge tank

Q_{CHP}: Heat recovered from a combined heat and power unit

Q_{demand}: Total energy demand

QIIME: Quantitative insights of microbial ecology

Q_T: Heating requirements

rDNA: Ribosomal deoxyribonucleotide

RNA: Ribonucleotide

SAOB: Syntrophic acetate-oxidising bacteria

S-COD: Soluble chemical oxygen demand

SEM: Scanning Electron Microscopy

SGD: Specific gas demand

SMP: Soluble microbial product

SRA: Sequence Reads Archive

SRB: Sulphate reducing bacteria

SRT: Solids retention time

TAN: Total ammonia nitrogen

TBMP: Theoretical biochemical methane potential

TCOD: Total chemical oxygen demand

TEC: Total eukaryotic cells

TN: Total nitrogen

TMP: Transmembrane pressure

TMP_{MAX}: Maximum transmembrane pressure

TP: Total phosphorus

TS: Total solids

TSS: Total suspended solids

U: Heat transfer coefficient

UASB: Anaerobic sludge blanket

UHF: Hollow-fibre ultrafiltration membrane

VFA: Volatile fatty acids

VS: Volatile solids

VSS: Volatile suspended solids

WAS: Waste activated sludge

W_{BG}: Electricity production from biogas

WL: Wood Ljungdahl

WP: Waste paper

WRRF: Water resource recovery facility

WT: Wastewater treatment

WWTP: Wastewater treatment plant

ΔG^0: Standard Gibbs-free energy

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APPENDIX: RESUMEN EXTENDIDO

Antecedentes

El continuo crecimiento de la población mundial ha provocado un aumento en el consumo de combustibles fósiles y en la generación de residuos municipales e industriales. Por este motivo, la mayoría de países está impulsando nuevas legislaciones que dirijan la investigación hacia la búsqueda de nuevas configuraciones para las estaciones depuradoras de aguas residuales (WWTP), estableciendo mayores restricciones para el vertido de efluentes líquidos y fango e imponiendo la necesidad de que estas WWTP sean energéticamente autosuficientes. Por tanto, se necesita un cambio de paradigma desde el tradicional tratamiento de aguas residuales hacia un nuevo sistema sostenible en el que las plantas convencionales de tratamiento de aguas residuales sean consideradas plantas de recuperación de recursos (WRRF).

La digestión anaerobia (AD) es parte de este nuevo concepto de WRRF ya que no se necesita oxígeno para la eliminación de las sustancias orgánicas, se producen menos biosólidos y los compuestos biodegradables se convierten en metano. Concretamente, los biorreactores anaerobios de membrana (AnMBRs) han aparecido como una alternativa viable a los tratamientos convencionales, ya que combinan la AD con la filtración por membrana, lo que ofrece varias ventajas: i) la disociación del tiempo de retención hidráulico (HRT) del tiempo de retención de sólidos (SRT), lo que evita que los microorganismos sean lavados ya que la biomasa se retiene en la membrana; ii) una gran eficiencia en cuanto a la eliminación de materia orgánica y sólidos en suspensión, lo que ofrece un efluente de alta calidad en el que también se han eliminado contaminantes microbianos (bacterias, microalgas, parásitos) (Robles et al., 2018); iii) potencial de recuperación de recursos; y iv) menores costes de energía.

El efluente del tratamiento AnMBR presenta un bajo contenido en sólidos, pero presenta altas concentraciones de nutrientes, que pueden recuperarse mediante el cultivo de microalgas. Estas microalgas podrían ser posteriormente digeridas anaeróbicamente, dando lugar a un lodo digerido enriquecido en nutrientes que podrían ser recuperados, completando un esquema en el que la energía y los nutrientes se recuperan a partir de las aguas residuales. Sin embargo, la AD de microalgas todavía presentan algunos

inconvenientes, como la alta resistencia a la biodegradación que presentan sus paredes celulares o el bajo ratio C/N de las microalgas (debido a su alto contenido de nitrógeno), que no es el óptimo para el proceso de AD (Giménez et al., 2018). Estos inconvenientes podrían resolverse añadiendo un co-sustrato rico en carbono, como residuos de papel o fango de depuradora, ya que este tipo de sustrato podría aumentar la relación C/N, evitando una posible inhibición por amoniaco y aumentando así la estabilidad del proceso y el rendimiento de la AD (Wang et al., 2013; Yen y Brune, 2007). Se han publicado diversas investigaciones sobre la co-digestión anaerobia (ACoD) de microalgas con diversos tipos de fangos (Mahdy et al., 2015; Olsson et al., 2014; Wang et al, 2013), pero se trata de ensayos realizados a corto plazo, que se basan en microalgas cultivadas con medios sintéticos o aguas residuales filtradas, y cuyos resultados podrían no ser fiables para escalar el proceso a escala industrial. Además, los ensayos de ACoD de microalgas publicados han evaluado el sistema trabajando en condiciones mesófilas, pero no en condiciones termófilas. El objetivo de la presente tesis es la evaluación a largo plazo de un sistema continuo de ACoD que trata microalgas sin pretratar (procedentes de una planta piloto de fotobiorreactores de membrana (MPBR) que trata aguas residuales) y fango primario.

Plan de tesis y objetivos

El objetivo general de esta tesis es la evaluación de la ACoD de fango primario y microalgas a largo plazo en un AnMBR para obtener recursos valiosos a partir del agua residual. Además, este trabajo tiene como objetivo estudiar la eficiencia de los procesos biológicos y de filtración, así como analizar la comunidad microbiana que lleva a cabo el proceso de co-digestión. Se estudiaron diferentes parámetros operacionales a escala laboratorio y las condiciones más recomendables se escogieron para escalar el proceso a escala piloto.

Dado que esta tesis se presenta como compendio de artículos, se ha estructurado en diferentes capítulos que recogen los resultados publicados. Los objetivos específicos de cada capítulo se describen a continuación:

Capítulo IV:

- Evaluar la ACoD de microalgas y fango primario a escala laboratorio, trabajando con SRT altos (100 d) y condiciones mesófilas (35 ºC).
- Analizar la estabilidad del proceso biológico y la comunidad microbiana en dos reactores mesofílicos conectados, actuando el segundo reactor como un reservorio de biomasa.
- Caracterizar la población microbiana involucrada en la ACoD: diferencias entre el inóculo y los dos reactores mesófilos.

Capítulo V:

- Evaluar la ACoD de microalgas y fango primario a escala laboratorio trabajando en condiciones termófilas (55 ºC).
- Evaluar la toxicidad del amoniaco en un reactor continuo de tanque agitado (CSTR) termófilo y en un AnMBR termófilo.
- Determinar el efecto sobre el proceso biológico y la población microbiana de una subida progresiva de la velocidad de carga orgánica (OLR).
- Determinar la incidencia de la implementación de una membrana en el sistema sobre el proceso biológico y sobre la población microbiana.

Capítulo VI:

- Evaluar la ACoD de microalgas y fango primario a escala laboratorio trabajando a 70 d de SRT y condiciones mesófilas (35 ºC) para saber la incidencia del SRT sobre la ACoD.
- Estudiar el escalado desde laboratorio a planta piloto de la ACoD de microalgas y fango primario trabajando en condiciones mesófilas.
- Llevar a cabo un balance de energía de la ACoD trabajando en condiciones mesófilas y termófilas. Comparar los resultados obtenidos con los resultados del capítulo V.
- Llevar a cabo un balance económico de la ACoD trabajando a un SRT de 100 o 70 d. Comparar resultados obtenidos con resultados del capítulo IV.
- Estudiar el proceso de filtración a escala laboratorio y piloto. Estudiar el efecto de diferentes parámetros sobre el proceso de filtración.

Capítulo VII:

- Evaluar la producción de biogás y la recuperación de nutrientes en el proceso de ACoD de microalgas y fango primario a escala piloto.
- Estudiar el proceso de compostaje a escala laboratorio. Comparar el proceso de compostaje de un fango digerido convencional y el fango obtenido en el proceso de ACoD de fango primario y algas.
- Estudiar diferentes condiciones operacionales sobre el proceso de compostaje.
- Evaluar la recuperación de nitrógeno y fósforo del permeado obtenido de la planta piloto de ACoD.

Materiales y métodos

Sustratos utilizados para los ensayos de co-digestión

Los sustratos utilizados para el estudio del proceso de ACoD fueron microalgas y fango primario. Las microalgas se obtuvieron de una MPBR situada en la WWTP de la Cuenca del Carraixet (González-Camejo et al, 2019). Esta planta MPBR se alimentaba con el efluente de un proceso previo de AD llevado a cabo en una planta piloto AnMBR, situada también en la WWTP del Carraixet (Giménez et al., 2011). El efluente del tratamiento AnMBR está libre de sólidos suspendidos y materia orgánica, pero presenta una concentración alta de nutrientes, por lo que, el cultivo de microalgas se utiliza para eliminar esos nutrientes. Dicho cultivo de microalgas estaba compuesto principalmente por las especies *Chlorella* y *Scenedesmus* (<99% del total de células eucariotas). Para alcanzar la OLR deseada en el proceso de ACoD, las microalgas se concentraron en un módulo externo de filtración tubular de flujo cruzado, conteniendo fibras de membrana de ultrafiltración (HF 5.0-43-PM500, ROMICON® Koch Membrane Systems, USA) con un tamaño de poro de 500 kDa y un área de filtración de 2,1 m^2. Por otro lado, el fango primario se obtuvo del espesador por gravedad de la WWTP del Carraixet y se filtró a través de un filtro de 0,5 mm para descartar grandes partículas. Los sustratos se caracterizaban y se almacenaban en la nevera durante un máximo de 3 semanas.

Montaje experimental y condiciones operacionales para los ensayos de co-digestión

El proceso de ACoD se estudió a escala laboratorio y piloto y el diseño experimental a ambas escalas fue muy similar.

- Operación a escala laboratorio (Capítulos IV, V y VI)

El proceso de ACoD a escala laboratorio se estudió en condiciones mesófilas y termófilas. El montaje experimental utilizado para el estudio de ambas temperaturas fue muy similar, con diferencias en el método de agitación y el tipo de membrana utilizado. El reactor anaerobio mesófilo 1 (AnMBR) estaba acoplado a un módulo de membranas de ultrafiltración de fibra hueca (PURON Koch membrane systems), con un tamaño de poro de 0,03 μm, permitiéndose así la disociación entre el SRT y el HRT. El reactor anaerobio mesófilo 2 (AnR) no estaba acoplado a membrana, manteniendo el SRT igual al HRT. Este reactor se mantuvo conectado al anterior, alimentándose con la purga diaria obtenida del AnMBR mesófilo. Tres soplantes aseguraron la agitación de los reactores y la membrana, manteniendo el cultivo homogéneo. El reactor anaerobio termófilo se puso en marcha bajo la configuración de CSTR, pero, tras 45 días de experimentación, se implementó una membrana cerámica de ultrafiltración (Likuid Nanotek, España) con un tamaño de poro de 0,1 μm. Este reactor se agitó mecánicamente.

Los tres reactores (mesófilos y termófilo) utilizados eran reactores cilíndricos (de metacrilato en el caso de los reactores mesófilos y de polipropileno en el caso del reactor termófilo), con un volumen útil de 9 L y se mantuvieron a la temperatura deseada (35 o 55 ºC) mediante una camisa de agua situada alrededor del digestor, que permite el paso de agua circulando desde un baño termostático de agua. Un contador de biogás MGC-1 milligas-counter (Ritter, Germany) se conectó a cada reactor para monitorizar la producción diaria de biogás. Además, los reactores estaban dotados de sondas de temperatura, pH y redox, que permiten el control de estos parámetros para saber cómo estaba evolucionando el proceso biológico.

Ambos AnMBRs, termófilo y mesófilo se alimentaron una vez al día con fango primario y microalgas en proporciones de 62% y 38%, respectivamente, basado en el contenido de sólidos volátiles (VS). Las condiciones operacionales estudiadas durante todo el periodo experimental se muestran en la Tabla 1.

Tabla 1. Condiciones experimentales estudiadas en los distintos reactores de laboratorio.

	AnMBR mesófilo		**AnR mesófilo**
Periodo	PI	PII	PI
SRT (d)	100	70	100
HRT (d)	30	30	100
OLR ($gCOD \cdot L^{-1} \cdot d^{-1}$)	0,52	0,5	0,15
Temperatura (ºC)	35	35	35
Membrana	Sí	Sí	No
Biomasa microalgal	*Chlorella* y *Scenedesmus*	*Chlorella*	*Chlorella* y *Scenedesmus*
Días de operación	440	390	440

			AnMBR termófilo		
Periodo	Start-up	PI	PII	PIII	PIV
SRT (d)	70	70	70	70	70
HRT (d)	70	30	30	30	30
OLR ($gCOD \cdot L^{-1} \cdot d^{-1}$)	0,22	0,17	0,30	0,40	0,50
Temperatura (ºC)	59	55	55	55	55
Membrana	No	Sí	Sí	Sí	Sí
Biomasa microalgal	*Chlorella*	*Chlorella*	*Chlorella*	*Chlorella*	*Chlorella*
Días de operación	0-45	46-253	254-423	424-471	472-700

- Operación a escala piloto (Capítulo VI)

El montaje experimental usado en planta piloto fue similar al utilizado en laboratorio. La planta piloto consistió en un co-digestor anaerobio de 1010 L de capacidad (hasta 910 L de volumen de trabajo y el resto espacio de cabeza), acoplado a un módulo de membranas de ultrafiltración de fibra hueca (0,03 µm tamaño de poro, PURON® KMS, USA) y 4 tanques auxiliares (almacenamiento de permeado, purga, trampa de espumas y homogeneización del alimento). En el tanque de homogeneización se mezcló el alimento mixto de cultivo de algas y fango primario espesado. El biogás producido se midió por medio de un contador de gas (BK-G4M, Elster, USA). Para mejorar las condiciones de agitación del codigestor y favorecer el *stripping* de los gases producidos desde la fase líquida, se recirculó una fracción del biogás producido hasta el digestor mediante una

soplante. Otra fracción del biogás producido se recirculó hasta la membrana para evitar la formación de biofilm. Para controlar la temperatura del digestor (35 ºC), se instalaron tres resistencias en la pared del reactor. La planta piloto de ACoD se operó durante 365 días a 70 d de SRT, 35 º C y una OLR de 0,5 g $COD \cdot L^{-1} \cdot d^{-1}$.

Operación de la membrana de filtración (Capítulo VI)

El proceso de filtración se estudió a escala laboratorio y piloto en condiciones mesófilas. En el AnMBR mesófilo a escala laboratorio, la membrana se operó de manera discontinua (se obtenía permeado una vez al día para mantener el HRT) durante los 360 primeros días de operación del PII (Tabla 1). Para evaluar la viabilidad de escalar el proceso de filtración a escala piloto, se realizaron experimentos de filtración durante los siguientes 30 días de experimentación, operando a un flujo de permeado (J_{20}) de 5,8 $L \cdot m^{-2} \cdot h^{-1}$ (LMH) y una demanda de gas específica (SGD) de 0,15 $N \cdot m^3 \cdot m^{-2} \cdot h^{-1}$. Los tiempos de filtración y relajación establecidos fueron 90 y 40 s, respectivamente. Con el objetivo de determinar posibles incrementos de la presencia de fouling o la aparición de fouling irreversible, la presión transmembrana (TMP) y la permeabilidad se evaluaron dos veces por semana operando a tres flujos diferentes (3,5; 7,0 y 10,5 LMH), coincidiendo con la determinación de productos solubles microbianos (SMPs).

A escala piloto, el proceso de filtración se evaluó durante todo el experimento y se realizaron ensayos específicos para determinar el efecto de distintos parámetros sobre la eficiencia en la filtración durante los últimos 100 d de experimentación. Un ciclo básico de relajación-filtración fue establecido para operar la membrana. Durante los primeros 260 d, la membrana se operó a un J_{20} de 3,5 LMH y un tiempo de filtración de 180 s. Durante los últimos 100 d se realizaron 4 experimentos en los que la membrana se operó a diferente SGD, tiempo de filtración y J_{20}. Se realizó una limpieza física de la membrana después de cada experimento. Las condiciones evaluadas en cada experimento se muestran en la Tabla 2.

Tabla 2. Condiciones experimentales de los 4 ensayos de filtración realizados a escala piloto.

	Demanda específica de gas ($N \cdot m^3 \cdot m^{-2} \cdot h^{-1}$)	**Flujo de permeado** ($L \cdot m^{-2} \cdot h^{-1}$)	**Ratio de recirculación**	**Tiempo de filtración** (s)
Experimento I	0,6244 ± 0,0005	5,6 ± 0,3	1,81 ± 0,00	300
Experimento II	0,151 ± 0,002	5,5 ±0,7	1,7 ± 0,2	160
Experimento III	0,15 ± 0,02	4,18 ± 0,04	1,92 ± 0,05	160
Experimento IV	0,32 ± 0,01	4,22 ± 0,03	1,81 ± 0,04	160

Montaje experimental para el estudio de recuperación de nutrientes (Capítulo VII)

- Estudio del proceso de compostaje

El estudio de compostaje del fango obtenido en la planta de ACoD se realizó a escala laboratorio. El proceso de compostaje de un fango convencional procedente de un digestor anaerobio industrial también se analizó para comparar los resultados obtenidos. Ambos fangos se pretrataron por centrifugación para eliminar el exceso de humedad, alcanzando valores alrededor del 80-87% de humedad. Para asegurar condiciones óptimas para el proceso de compostaje, se añadieron restos de poda, que actuaron como agente estructurante (BA), a las mezclas de fango. Las mezclas entre BA y fango se realizaron siguiendo dos métodos diferentes: proporciones teóricas o proporciones volumétricas. Las proporciones volumétricas se realizaron añadiendo 2,5 volúmenes de BA por cada volumen de fango. Las proporciones teóricas se calcularon fijando la ratio C/N de la mezcla en un valor de 25 y el valor del contenido en humedad entre 50% y 70%.

Los experimentos se llevaron a cabo en 7 reactores (R_{A1}-R_{A4}; R_{C1}-R_{C3}) Dewar (KGW Isotherm, Alemania), con un volumen de 4 L, aislados térmicamente, especialmente diseñados para realizar un estudio de compostaje. Se incorporó una malla de plástico en el fondo de los reactores y se cubrió con una capa de grava para permitir una correcta separación entre el lixiviado y el material compostado. Se colocó una sonda de temperatura en cada reactor para la medición diaria de la temperatura.

Los reactores se operaron en pares, durante un máximo de 44 días, para evaluar el efecto de diferentes parámetros sobre el proceso de compostaje. Se evaluaron tres parámetros: i) aireación, ii) adición de un inóculo (obtenido de la planta industrial de compostaje de Vintena) y iii) mezcla entre el BA y el fango. La Tabla 3 muestra las condiciones operacionales estudiadas en cada reactor.

Tabla 3. Condiciones operacionales para los 7 reactores de compostaje.

Nombre del reactor	Días en operación	Fango	Proporción de la mezcla	Aireación	Inóculo
R_{A1}	36	ACoD	Teórica	Forzada	✓
R_{C1}	36	Convencional	Teórica	Forzada	✓
R_{A2}	44	ACoD	Volumétrica	Volteado	✓
R_{C2}	44	Convencional	Volumétrica	Volteado	✓
R_{A3}	27	ACoD	Teórica	Volteado	✓
R_{C3}	27	Convencional	Teórica	Volteado	✓
R_{A4}	31	ACoD	Volumétrica	Volteado	-

- Estudio de recuperación de amonio

Para la recuperación de nitrógeno del permeado obtenido en la planta piloto de ACoD, se usó un contactor de membrana hidrofóbica de polipropileno de fibra hueca (HFMC) (X50 2.5x8 Liqui-Cel®, USA) a escala laboratorio. La membrana HFMC, de una superficie de 1,4 m^2, estaba conectada a dos tanques de 1,2 L en los que el permeado y la solución ácida se almacenaron. La solución ácida (0,05M H_2SO_4) circulaba en la parte interior de la fibra a un caudal de 0,4 $L \cdot min^{-1}$ mientras que el permeado se alimentó en la parte exterior a un caudal de 0,6 $L \cdot min^{-1}$ y un pH de 10.

Técnicas empleadas

Con el fin de controlar el proceso de AD, se analizaron las corrientes de alimentación y purga de todos los reactores, así como las corrientes de permeado obtenidas, tanto a escala laboratorio como a escala piloto. Se analizaron los siguientes parámetros, por duplicado, tres veces por semana: VS, sólidos totales (TS), ácidos grasos volátiles (VFA) y alcalinidad (Alk). Además, una vez por semana, se determinaron otros parámetros como: demanda química de oxígeno total y soluble (COD y COD-S, respectivamente), sólidos suspendidos totales (TSS), sólidos suspendidos volátiles (VSS), nitrógeno total (TN),

amonio (NH_4-N), fósforo total (TP), fosfato (PO_4-P) y sulfato (SO_4-S). Todas las analíticas se realizaron en base a *Standard Methods for the Examination of Water & Wastewater* (APHA, 2005), exceptuando la medición de alcalinidad y AGV, que se determinaron por titriación mediante el método recomendado por *South African Water Research Commission* (Moosbrugger *et al*., 1992).

Además, se evaluó el rendimiento del sistema a través de la medida del porcentaje de metano presente en el biogás mediante un Cromatógrafo de Gases (GC-FID, Agilent Technologies, EE.UU.). El contenido en C, N, H y S de microalgas y fango primario fue analizado por espectroscopia de rayos X por medio de un XL-30ESEM (Philips, Holanda).

Para evaluar el proceso de filtración en el AnMBR mesófilo a escala de laboratorio, se midió la viscosidad con un viscosímetro de Cannon-Fenske (Serie 200, Merck, Alemania) y el contenido de SMP (atribuido únicamente a las concentraciones de proteínas y carbohidratos). La concentración de carbohidratos se determinó mediante el método Dubois (Dubois et al., 1956). La concentración de proteínas se determinó utilizando un kit comercial de proteína total, Micro Lowry, Modificación de Peterson (Sigma-Aldrich, EE.UU.), basado en el método Lowry-Peterson (Peterson, 1977).

Para evaluar la eficiencia del proceso de compostaje, se midió la ratio C/N, el contenido en materia orgánica, la porosidad y el contenido en TN en las muestras de compost y en las muestras de BA. Además, se evaluó el contenido en patógenos, *E. coli* y *Salmonella* spp., siguiendo los métodos UNE-EN ISO 9308-1:2014 y UNE-EN ISO 19250.

Caracterización de la comunidad microbiana

La población microbiana se analizó en los reactores de laboratorio: AnMBR y AnR mesófilos y en el reactor termófilo (Capítulos IV y V). En los reactores mesófilos se tomaron muestras del inóculo y de ambos reactores durante el periodo PI y se extrajo el DNA siguiendo el protocolo descrito por Zamorano-López et al. (2019) (Capítulo IV). En el reactor termófilo se extrajeron muestras durante la puesta en marcha y durante los 4 periodos evaluados y se extrajo el DNA utilizando el kit E.Z.N.A DNA Extraction Kit for Soil (Omega-Biotek, USA) (Capítulo V). El análisis del gen 16S rRNA de bacterias y arqueas de todos los reactores se realizó mediante secuenciación de amplicones. Las bibliotecas se prepararon utilizando cebadores específicos para la región hipervariable

v3-4 del gen diana. La secuenciación se realizó en un secuenciador Illumina MiSeq del servicio de secuenciación FISABIO (Valencia, España). Las secuencias sin procesar resultantes se depositaron en la plataforma NCBI.

La biodiversidad en los reactores mesofílicos y termófilos se calculó mediante los índices: Shannon-Wiener y Simpson, que determinan la diversidad alfa teniendo en cuenta la riqueza de las especies. Se realizó un análisis de coordenadas principales (PCoA) basado en la matriz de distancias Bray-Curtis para explorar la diversidad beta de las diferentes muestras recogidas de los AnMBR y AnR.

Resultados

El proceso de ACoD de microalgas y fango primario se estudió primero a escala laboratorio, evaluando diferentes parámetros (SRT, temperatura, implementación de membrana al sistema y OLR). Tras conocer la incidencia de dichos parámetros sobre el proceso biológico y la comunidad microbiana, se realizó un balance de energía y un balance económico para elegir las condiciones más recomendables para escalar el proceso a escala piloto.

<u>Operación de co-digestión anaerobia de microalgas a escala laboratorio</u>

- Efecto del tiempo de retención de sólidos (Capítulos IV y VI)

Se estudiaron dos periodos trabajando a diferentes SRT (100 d y 70 d) en el AnMBR mesófilo de laboratorio, que se operó durante 830 d. Durante estos dos periodos, el HRT y la OLR se mantuvieron constantes (30 d y 0,5 $gCOD \cdot L^{-1} \cdot d^{-1}$, respectivamente), por tanto, las diferencias encontradas en la eficiencia del proceso de AD pueden ser atribuidas al diferente SRT al que se trabajaba. El sistema se mostró muy estable con respecto a valores de alcalinidad, producción de VFA y pH, trabajando a ambos SRT. Sin embargo, se observó una mayor producción de metano trabajando a 100 d de SRT. Se observaron rendimientos de metano de 256 $mLCH_4 \cdot gCOD_{inf}^{-1}$ (435 $mLCH_4 \cdot gSV_{inf}^{-1}$) y 225 $mLCH_4 \cdot gCOD_{inf}^{-1}$ (391 $mLCH_4 \cdot gTVS_{inf}^{-1}$) trabajando a 100 y 70 d de SRT, respectivamente, lo que se corresponde con porcentajes de biodegradabilidad de 73,1 y 64,6%. Por tanto, se puede alcanzar una producción de metano 14% mayor trabajando con un SRT 30 d superior, ya que se promueve una mayor actividad hidrolítica y metanogénica. Estos resultados muestran concordancia con Greses (2017) y Giménez et

al. (2017) quienes observaron también que se obtenían mayores rendimientos de metano trabajando a un SRT mayor digiriendo microalgas en un reactor AnMBR.

El efecto de doblar el SRT también se estudió con el segundo reactor mesófilo (AnR) conectado al primero (AnMBR), durante el periodo PI. En este segundo reactor, el HRT era igual al SRT (debido a la ausencia de membrana). Por tanto, el SRT total del sistema era de 200 d (100 d en el AnMBR y 100 d en el AnR). El AnR alcanzó un rendimiento de metano de 35 $mLCH_4 \cdot gCOD_{inf}^{-1}$ (biodegradabilidad del 10.2%). El porcentaje de biodegradabilidad total obtenido con los dos sistemas fue del 78%.

- Efecto de la temperatura (Capítulos V y VI)

El efecto de la temperatura se estudió a escala laboratorio operando dos AnMBR a 35 o 55 ºC, manteniendo constantes el resto de condiciones operacionales. Se observó un mayor rendimiento de metano trabajando a condiciones termófilas: trabajando a 35 ºC se obtuvieron 225 $mLCH_4 \cdot gCOD_{inf}^{-1}$ (391 $mLCH_4 \cdot gVS_{inf}^{-1}$), mientras que trabajando a 55 ºC se obtuvieron 242 $mLCH_4 \cdot gCOD_{inf}^{-1}$ (429 $mLCH_4 \cdot gSV_{inf}^{-1}$). Zamalloa et al. (2012) encontraron un incremento de 1,3 veces en la producción de biogás al trabajar a 55 ºC comparando con la operación a 35 ºC digiriendo *Scenedesmus* en un CSTR. La mayor producción de metano se debe a una mayor actividad hidrolítica, y una mayor degradación de compuestos intermedios que permite incrementar la producción de biogás.

- Efecto de implementar una membrana al sistema (Capítulo V)

El reactor termófilo de laboratorio se puso en marcha utilizando una configuración CSTR, por tanto, el HRT era igual al SRT (70 d). Tras varios días de operación a una carga de 0,22 $gCOD \cdot L^{-1} \cdot d^{-1}$ se observó que no se estaba produciendo metano y que los VFA producidos se estaban acumulando, por lo que el sistema no los estaba degradando. La concentración de amoniaco medida en ese momento fue de 29,9 $mgNH_3\text{-}N \cdot L^{-1}$ por lo que se asume que este valor puede inhibir un sistema de ACoD de microalgas trabajando en un sistema CSTR. Al implementar la membrana al sistema, la acumulación de VFA fue desapareciendo y la producción de metano aumentando, llegando a alcanzar una biodegradabilidad del 51.4% trabajando a 0,17 $gCOD \cdot L^{-1} \cdot d^{-1}$ sin observar inhibición del sistema, por lo que la implementación de la membrana en el sistema permitió la operación termófila del reactor.

- Efecto de la velocidad de carga orgánica (Capítulo V)

En el AnMBR termófilo de laboratorio se estudió un incremento progresivo de la OLR desde 0,17 a 0,5 $gCOD \cdot L^{-1} \cdot d^{-1}$. El incremento de la carga provocó ligeras acumulaciones de VFA y TS al inicio de cada periodo (cada vez que se aumentaba la carga) pero estas acumulaciones desaparecían con el tiempo, alcanzando altas eficiencias de eliminación de TS y biodegradabilidades. El aumento de 3 veces la OLR conllevó un aumento en la producción de metano del 35%, alcanzando el sistema una biodegradabilidad del 69% y una estabilidad que no se observó trabajando en un CSTR.

- Caracterización de la población microbiana (Capítulos IV y V)

La caracterización de la población microbiana encargada de llevar a cabo el proceso de ACoD se estudió en los AnMBR de laboratorio, mesófilo y termófilo. Los filos mayoritarios observados fueron Chloroflexi, Coprothermobacteraota, Thermotogae, Hydrothermae, Proteobacteria, Firmicutes, Caldiserica, Cloacimonetes, Bacteroidetes, Actinobacteria y Synergistetes. Se observó un núcleo microbiano común en ambos reactores (mesófilo y termófilo) formado por bacterias degradadoras de polisacáridos y proteínas, debido al alto contenido en proteínas y celulosa de los sustratos degradados (microalgas y fango primario). Algunos microorganismos hidrolíticos, encargados de dicha degradación de polisacáridos y proteínas (*Defluviitoga* y *Coprothermobacter*, respectivamente) y metanógenos (*Methanosaeta*) estaban presentes en ambos reactores. Microorganismos hidrolíticos característicos de reactores termófilos como *Fervidobacterium* o *Thermogutta*, entre otros, se encontraron únicamente en el reactor termófilo y microorganismos característicos de reactores mesófilos como miembros de la familia Anaerolineaceae o *Mesotoga*, entre otros, fueron observados solo en el reactor mesófilo. En ambos reactores se observó alta abundancia de grupos sintróficos: *Smithella* en el caso del reactor mesófilo y *W5* en el caso del reactor termófilo. Estos microorganismos han sido descritos como oxidadores de propionato, dando lugar a acetato e hidrógeno y, por tanto, manteniendo relaciones de sintrofia con metanógenos aceticlásticos e hidrogenotróficos. Otros microorganismos como *Defluviitoga* y *Acetomicrobium*, encontrados en los reactores, también han sido descritos como microrganismos sintróficos. Por tanto, estas relaciones de sintrofia tienen un papel clave en la co-digestión de microalgas y fango primario.

El estudio comparativo de las muestras de inóculo y las muestras del reactor mesófilo (Capítulo IV) permitió observar el cambio producido en la comunidad microbiana. El inóculo utilizado provenía de un reactor digiriendo algas como único sustrato, por lo que algunos microorganismos presentes en el inóculo, característicos de la degradación de algas, como miembros del filo Chloroflexi y Cloacimonetes, se observaron también en el co-digestor. Sin embargo, el hecho de añadir un nuevo sustrato (fango primario) provocó la aparición de microorganismos no presentes en el inóculo y potenció el desarrollo de redes sintróficas como se ha comentado en el párrafo anterior.

El estudio de la comunidad microbiana realizado en el reactor termófilo (Capítulo V) determinó que el incremento de OLR realizado en el reactor provocó cambios en las abundancias relativas de los microorganismos, pero manteniéndose la diversidad existente. El estudio en este reactor también permitió observar que la implementación de la membrana al sistema provocó un cambio en la población microbiana, ya que la diversidad aumentó tras incluir la membrana en el sistema y aparecieron nuevos grupos que no estaban presentes en el CSTR. Este resultado también se observó al estudiar los dos reactores mesófilos (AnMBR y AnR) (Capítulo IV), ya que la diversidad observada en el AnMBR fue mayor a la observada en el AnR.

Operación de co-digestión anaerobia de microalgas a escala piloto

Los balances económicos y de energía realizados en base a los resultados obtenidos en el laboratorio mostraron que las condiciones operacionales recomendadas para operar la planta piloto eran 70 d de SRT y 35 ºC. La planta piloto fue operada durante un año trabajando a dichas condiciones. El sistema mostró buenos rendimientos, alcanzando altas eficiencias de producción de metano y eliminación de sólidos y materia orgánica. Se produjeron 78 $L_{biogas} \cdot d^{-1}$, lo que corresponde con 154 $mL_{biogas} \cdot d^{-1} \cdot L_{reactor}^{-1}$. Por tanto, el co-digestor anaerobio alcanzó un rendimiento de metano de 215 $mL_{CH_4} \cdot gCOD_{inf}^{-1}$ (370 $mL_{CH_4} \cdot gTVS_{inf}^{-1}$) y una eficiencia en la eliminación de VS del 64%. El sistema mostró también estabilidad ya que los VFA producidos se convirtieron en metano sin producirse acumulación de los mismos. Los resultados obtenidos a escala piloto fueron muy similares a los obtenidos en laboratorio, indicativo de la reproducibilidad del sistema, aspecto necesario para un posible escalado a escala industrial.

Experimentos de filtración

Se realizaron experimentos de filtración en el reactor mesófilo tanto a escala laboratorio como a escala piloto. Los resultados a escala de laboratorio indicaron que el sistema era estable durante los 30 d que duró la experimentación, trabajando a un J_{20} promedio de 5,8 LMH, manteniendo los valores de permeabilidad de la membrana alrededor de 35 $LMH \cdot bar^{-1}$. El valor de permeabilidad de la membrana obtenido es bajo, posiblemente debido a la viscosidad del licor mezcla (alrededor de 1,5 cP), pero se mantuvo estable durante 30 d, lo que sugiere que no se esperaba la aparición de fouling irreversible en la operación de la membrana a largo plazo. El análisis de flujo a corto plazo mostró resistencias similares de filtración durante el experimento, incluso a flujos relativamente altos (7-10,5 LMH), lo que corroboró la hipótesis de que no se esperaban problemas de fouling irreversible. La concentración de SMP en el reactor también se mantuvo estable durante el experimento, sin observarse una acumulación.

Se llevaron a cabo experimentos de filtración a escala piloto para encontrar el funcionamiento óptimo de la membrana y conocer su comportamiento a largo plazo. Los resultados de los cuatro experimentos con diferentes SGD, tiempo de filtración y J_{20} indicaron que operar a un J_{20} de 4,2 LMH, tiempo de filtración de 160 s y SGD de 0,15 $N \cdot m^3 \cdot m^{-2} \cdot h^{-1}$ parece ser lo recomendado para el funcionamiento de un proceso de filtración asociado a la ACoD de microalgas y fango primario.

Recuperación de nutrientes

Durante el proceso de ACoD se obtuvieron efluentes (permeado y digestato) ricos en nutrientes. Alrededor de 400 $mgNH_4\text{-}N \cdot L^{-1}$ y 37 $mgPO_4\text{-}P \cdot L^{-1}$ se liberaron durante el proceso de ACoD. El contenido de amonio en el permeado fue mayor al contenido presente en el afluente, pero, en el caso del fosfato, la concentración en el permeado fue menor que en el afluente. Esto sugiere que procesos de precipitación estaban ocurriendo durante el proceso de AD. Por este motivo, se llevó a cabo un balance de P y los resultados obtenidos indicaron que un 74% del P estaba precipitando. Por tanto, solo el 26% estaría disponible para ser recuperado, por lo que el estudio experimental de recuperación de P no se llevó a cabo. Sin embargo, sí se llevó a cabo el estudio de recuperación de N por medio de un HFMC, a escala laboratorio. Se recuperó el N con una eficiencia del 99%.

Se obtuvo una solución de sulfato de amonio que podría ser utilizada como fertilizante comercial.

Por otro lado, se estudió el proceso de compostaje a escala laboratorio. Se obtuvo material estable (con índices respirométricos por debajo de 0,5 $mgO_2 \cdot g$ materia orgánica$^{-1} \cdot h^{-1}$ a 37 ºC) e higienizado (libre de *E. coli* y S*almonella* spp.) en dos de los 7 reactores estudiados, en los cuales se alcanzó la temperatura termófila. En dichos reactores, se habían añadido 400 mL de inóculo y, además, la mezcla entre el fango y el BA se realizó por el método de proporciones volumétricas (añadiendo 2,5 volúmenes de BA por cada volumen de fango), sugiriendo que estas dos condiciones han de tenerse en cuenta a la hora de realizar el proceso de compostaje. En los dos reactores en los que se obtuvo material higienizado no se obtuvo la máxima eliminación de materia orgánica y ratio C/N, sugiriendo que algunos factores como la aireación podrían optimizarse para obtener mejores resultados.

Discusión general

El proceso de ACoD de microalgas y fango primario se escaló con éxito desde escala de laboratorio a escala piloto y se estudió durante un año continuo. Este escalado es un indicador de la buena reproducibilidad del sistema, siendo este el primer paso frente a un futuro escalado industrial. Además, varios problemas que no ocurren a escala de laboratorio podrían ocurrir a escala piloto, trabajando con un volumen de reacción mucho mayor, equipos más complejos y de mayor tamaño y automatización del sistema. Encontrar esos problemas (por ejemplo, las fugas de biogás o el ensuciamiento de la membrana) ayuda a saber cuáles son los puntos débiles del equipo utilizado o del proceso en sí y resolverlos para futuras aplicaciones.

El efecto sinérgico que se produce al co-digerir las microalgas y el fango primario permite una mayor degradación de los sustratos que la que se obtiene al digerir los sustratos por separado, ofreciendo una alta eficiencia en la eliminación de materia orgánica y en la producción de metano. Los resultados de la caracterización microbiana mostraron que se produjeron relaciones sintróficas entre los microorganismos, dando lugar a una comunidad microbiana estable, capaz de degradar ambos sustratos. La alta estabilidad observada en el sistema y la elevada biodegradabilidad se asoció no sólo con el hecho de que la codigestión de ambos sustratos ofrecía efectos de sinergia sino también con el

hecho de que la implementación de una membrana al sistema ofrecía varias ventajas. Se logró la disrupción de las microalgas *Chlorella* o *Scenedesmus* sin necesidad de aplicar costosos pretratamientos, lo que hace que el proceso de ACoD en un AnMBR sea una alternativa prometedora que ahorra costes y recicla recursos.

Se recuperaron recursos útiles (metano, nutrientes y agua) de la planta piloto de ACoD. Como el AnMBR permitió obtener efluentes líquidos y sólidos, los recursos se recuperaron a través de tres corrientes: biogás, permeado y digestato. La planta piloto de ACoD forma parte de un esquema sostenible en el que se están eliminando los compuestos orgánicos de las aguas residuales crudas mediante un proceso de AD; los nutrientes del efluente de la AD se están eliminando mediante el cultivo de microalgas (ofreciendo un efluente líquido que cumple con los límites de vertido); y la ACoD final de las corrientes de desecho (microalgas y fango primario) se utiliza para la producción de biogás y subproductos ricos en nutrientes. Este esquema engloba tecnologías verdes en un escenario dentro del marco de economía circular para el tratamiento del agua residual que es muy necesario hoy en día para disminuir el uso de tecnologías que consumen recursos.

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