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

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Abstract

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

were observed in the microbial composition during anaerobic microalgae digestion when feeding only *Chlorella* or *Scenedesmus*. This is the first work revealing the composition of complex communities for semi-continuous bioenergy production from WRRF streams. The stability and maintenance of a microbial core over-time in semi-continuous AnMBRs is here shown supporting their future application in full-scale systems for raw microalgae digestion or co-digestion.

Keywords
16S rRNA gene; anaerobic digestion; AnMBR; biogas; codigestion; microalgae

1. Introduction

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

The biochemical composition of microalgae makes them suitable for bioenergy production through anaerobic digestion processes (Klassen et al., 2016). However, pretreatments used to improve their biodegradability are expensive making the methane production from microalgae unfeasible (Carrillo-Reyes et al., 2016). Therefore, feasible bioenergy generation from microalgae in future WRRFs needs biological strategies for microalgae cell disruption and degradation of the hydrolysed components. Raw conversion of

56 microalgae into biogas is possible when applying high solids retention times (SRT) in
57 continuous bioreactors under mesophilic and thermophilic conditions (Greses et al., 2018;
58 Klassen et al., 2016). As early reported by Zamalloa et al. (2012), the Anaerobic Membrane
59 Bioreactor (AnMBR) allows to increase the biomass retention whilst maintains low hydraulic
60 retention times (HRT), making possible the continuous anaerobic digestion of microalgae.

61 As early remarked by Rivière et al. (2009), the definition of microbial cores in
62 engineering systems can provide valuable information during operational parameter
63 optimization processes. Zamalloa et al. (2012) was the first work relying on microbial groups
64 of microalgae anaerobic digestion through 16S rRNA gene fingerprinting. More recently,
65 saccharolytic hydrolyzers and fermenters, as well as proteolytic bacteria from Bacteroidetes
66 and Firmicutes phyla have been identified during *Chlamydomonas reinhardtii* anaerobic
67 digestion (Klassen et al., 2017). However, differences in the microalgae species can lead to
68 different microalgae-degrading communities as their composition varies among their
69 phylogeny (Baudelet et al., 2017). Moreover, common microalgae that grow over sewage or
70 anaerobic effluents have more resistant cell walls and can therefore require higher
71 microbiological hydrolytic potentials. In this context, acclimation of anaerobic sludge is a
72 necessary step prior to continuous conversion of microalgae harvested from sewage-related
73 streams into biogas in WRRFs (Gonzalez-Fernandez et al., 2018). The effect of the type of
74 microalgae over the acclimated microbial community structures has not been thoroughly
75 explored yet in the literature as most of the studies are focused on a single microalga.

76 The longer the SRT, the more favorable environment for slow-growing microorganisms
77 that might be able to disrupt the microalgae cell walls (Greses et al., 2017). However, more
78 efficient biomethanization of microalgae could be obtained with more balanced C:N ratios
79 through the addition of a co-substrate with a high carbon content. The protein content of
80 microalgae has an important drawback as the degradation of these compounds results in the

release of nitrogen forms that can accumulate in anaerobic systems as free ammonia. Methanogens are sensitive to free ammonia and therefore, strategies to mitigate this inhibition risk are needed to enhance continuous energy production. According to Sialve et al. (2009), mass ratios between 20 and 35 have a positive effect over methane yield as well as over microalgae anaerobic digestion and mitigate the inhibition risk.

The favorable effect of co-digestion for microalgae anaerobic digestion was recently reported by Solé-Bundó et al. (2019). The authors achieved a 65% improved biomethanization when combining primary sludge and *Chlorella* biomass streams from a wastewater treatment plant. Also, the degradation of *Scenedesmus* with pig manure resulted in a 50% increase of the methane yield (Astals et al., 2015). Unfortunately, these studies did not evaluated the effect of SRT over microalgae co-digestion, despite the importance of this parameter to achieve high microalgae disruption rates (Greses et al., 2018). Also, although several studies have explored different microalgae co-digestion scenarios (Herrmann et al., 2016; Mahdy et al., 2014; Solé-Bundó et al., 2018), none of them have been performed in a semi-continuous system operated under high SRT. Solé-Bundó et al. (2019) reported a 330 mL CH_4·gVS production from *Chlorella* and primary sludge in continuous stirred tank reactors (CSTR) but they applied a low SRT of 20 days and a protease treatment to the microalgae biomass. Furthermore, the microbiological aspects were not explored in the abovementioned systems and hence, there is a lack of knowledge on the different groups involved in microalgae co-digestion compared to a single digestion. Only Li et al. (2017) reported the dominance of Bacteroidetes, Proteobacteria, Firmicutes and Spirochaetae during co-digestion of *Chlorella* and chicken manure. However, this study applied a pre-treatment of the microalgae. As reported by Córdova et al. (2018), microalgae pre-treatment leads on important changes in microbial patterns, functionality, strategies and interactions during microalgae anaerobic digestion. According to these authors, delta and gamma Proteobacteria were dominant for untreated

106 *Chlorella* biomass digestion, but Clostridia was the most important group after applying an
107 alkali-treatment to the same algal biomass. On the other hand, some of the co-substrates that
108 can be added during microalgae digestion (*e.g.* primary and secondary sludge or manure)
109 commonly have an inner microbial diversity that can disturb the microbial core developed
110 during microalgae degradation. These aspects need to be evaluated in continuous systems to
111 advance towards the design of management tools based on microbial community composition,
112 like specific biomarker monitoring, in bioenergy production systems.

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

130 **2. Materials and Methods**

2.1. *Bioreactor operational conditions*

Two different lab-scale mesophilic AnMBRs were operated to produce biogas from microalgae under the operational conditions summarized in Table 1. Both reactors were operated under mesophilic conditions (35°C). The first AnMBR (digester, Figure S1a) had a 12.4 L volume, 9.9 L working volume, considering the tank and the external hollow-fibre ultrafiltration membrane tank (0.42 m^2 surface, 0.05 µm pore size, PUR-ON® Koch Membrane Systems). The second AnMBR (co-digester, Figure S1b) had a 14 L volume (9 L working volume) and was equipped with an identical external membrane tank to the first AnMBR. A reservoir tank was coupled to the co-digester AnMBR and used for microbial analysis purposes as detailed later. The digester was inoculated with mesophilic sludge from a full-scale digester located in the municipal WTTP Carraixet (València, Spain). The co-digester was inoculated with the stored biomass from the digester, available in the reservoir.

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

2.2. *Feedstock sources*

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

157 microscopic observation and quantification (Pachés et al., 2012), during the first 24 months
158 more than the 90% of the phytoplankton observed in the MPBR were identified as *Scenedesmus*
159 spp. Later, a shift in the microalgae population of the MPBR occurred and instead more than
160 90% of the cells were *Chlorella* spp. This microalga was dominant in the MPBR for the 8
161 remaining months.

162 A cross-flow ultrafiltration hollow-fiber membrane unit (HF 5.0-43-PM500, PURON®
163 Koch Membrane Systems) was used for microalgae harvesting and concentration to the
164 required values prior to feed the AnMBRs to an organic loading rate (OLR) of 0.2-0.4 $gVS \cdot L^{-1} \cdot d^{-1}$
165 (see Table 1). Microalgae feedstock was prepared in a single batch for both systems and
166 then adjusted to the different concentrations for single- or co-digestion. The primary sludge
167 was collected from the gravity thickener, sieved through an aperture of 0.5 mm sieve and
168 diluted to 22.8 $gCOD \cdot L^{-1}$ to feed the AnMBR co-digester according to Table 1 OLR conditions
169 (62%-38% proportion of primary sludge and microalgae based on gVS determination). The
170 physicochemical characterization of feedstock samples was performed according to APHA
171 (2012) standard procedures. Feedstock sources were separately stored at 4°C (for no longer
172 that 3 weeks) to preserve its characteristics and avoid degradation.

173 ### 2.2. Performance analysis

174 Physicochemical analysis and biogas production were carried out per triplicate and three
175 times a week as in a previous study (Zamorano-López et al., 2019a). At least the data retrieved
176 during three pseudo-steady state weeks were considered to calculate the methane yield, the
177 biodegradability, the solids content of the system (in terms of total suspended solids, TSS) and
178 the total COD (TCOD). The methane yield was calculated on a COD basis, considering the
179 COD of the methane produced and measured in the biogas over the total influent COD
180 associated to each feedstock scenario. The biodegradability of the system was thus calculated

on this basis using the theoretical potential of 350 $mLCH_4 \cdot gCOD_{inf}^{-1}$ (TMP 0ºC, 1 atm) and expressed as the percentage of the biomethanization achieved for each feedstock scenario.

2.3. *Sample collection for microbial ecology analysis*

Digestate samples were extracted from each AnMBR during the different pseudo-steady state periods achieved for the different combination of operational parameters applied to each AnMBR (Table 1). Since pseudo steady state was reached before each biomass collection point, samples can be considered biological replicates for each microalgae mono- and co-digestion scenario evaluated. Under each period, stabilized measures of digestate COD and TSS in the digestate, as well as the methane yield were determined in each AnMBR (Table 2).

All samples were frozen at -20°C prior to the nucleic acid extraction. At least two samples were collected for each AnMBR experimental period regardless of the inoculum. In total, 13 samples were collected from the digester, whereas 9 samples were extracted from the co-digester. Co-digester samples were duplicated as the AnMBR co-digester set-up (Figure S1b) included a reservoir tank were the digestate extracted to maintain the SRT in the main tank was stored also at 35°C. Additionally, 9 samples were extracted from the reservoir at the same collection points than the co-digester. Two extra samples were also stored from the reservoir at days 124 and 170. Hence, 33 samples were used in total in this study for microbial analysis.

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

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

bioproject PRJNA434206, accession numbers SAMN11567542-50 (co-digester), SAMN11567551-63 (digester) and SAMN11567566-76 (reservoir).

2.5. *Diversity analysis*

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

2.6. *Biostatistics*

All biostatistics analysis were performed using R-studio (v.3.2) within vegan and mixomics packages. A principal co-ordinate analysis (PCoA) based on the weighted unifrac distances matrix was used to evaluate the beta-diversity of the different samples collected from both AnMBRs. Adonis test over the PCoA results were performed using 999 permutations for feedstock and digester categorical variable clusters. A Partial Least Square Discriminant Analysis (PLS-DA) was performed over all samples (digester, co-digester and reservoir) to explore the effect of the primary sludge addition over the AnMBRs populations. This statistical analysis allows to extract the most discriminant $OTU_{0.97}$ among a group of samples and their major association to any of the two AnMBR systems studied here.

3. Results and discussion

3.1. *16S rRNA sequencing data analysis and alpha-diversity measurements*

The 16S rRNA gene amplicon sequencing approach resulted in a total of 1,431,467 raw sequences that after downstreamanalysis with high-quality settings resulted in an average of

57,409 clean sequences per sample. After rarefaction to the minimum value of clean sequences observed in the dataset (27,647) different alpha diversity estimators were extracted (Table 3). To compare these values, only samples taken under the same SRT in each AnMBR scenario were considered, since this parameter can strongly enhance species richness and diversity in anaerobic systems with high solids retention capacity such as the AnMBR.

The highest diversity was found in the samples taken during *Chlorella* digestion: 4150 observed OTUs. This scenario also presented the highest diversity in terms of non-detected OTUs, which are estimated through the chao1 index (7075). On the contrary, the *Scenedesmus* scenario presumably had the minimum diversity observed with 3358 OTUs and an estimated 6023 chao1 index value. This could be related to the development of a more specific community for *Scenedesmus* digestion than for *Chlorella digestion*. As it has been reported in the literature, *Scenedesmus* is among the hardest Chlorophyta member for direct disruption using microbial communities due to the presence of algaenan (Fernandez et al., 2018). Although *Chlorella* cell walls are also composed of recalcitrant compounds similar to chitin (Baudelet et al., 2017), the n-alkaenan composition of algaenan could have a stronger selective pressure effect over microbial communities and therefore decrease AnMBR alpha diversity.

Phylogenetic similarity of each sample can be measured through the PD_whole_tree estimator (Table 3). The higher number of phylogenetic tree branches, the higher value of PD_whole_tree estimator and thus, this value reveals the existence of more diverse and distant species in each sample. The highest PD_whole_tree values were observed during *Chlorella* digestion, again suggesting that this was the more diverse feedstock scenario of the four studied. Between the two co-digestion scenarios, slight differences were observed in the three indexes (observed_otus, chao1 and PD_whole_tree). This could be related to the higher presence of microbial groups with wider metabolic capacities in the digester when both substrates were present than when it was only fed with microalgae.

The evenness measurement retrieved for each scenario (see simpson evenness index in Table 3) reflected that the changes in the relative abundance patterns of the observed OTUs were more dynamic in the co-digestion scenarios than when only microalgae was digested. It should be noticed that from an ecological perspective, the addition of a co-substrate which has a certain microbial diversity can enhance richness and evenness diversity due to the presence of minor and rare groups that might not be active in the anaerobic system but are though retained. Related to this, Chen et al. (2019) observed higher diversity in the primary sludge than in the anaerobic digester samples. Thus, primary sludge could also have enhanced evenness in the AnMBR co-digester in this work. Interestingly, Greses et al. (2017) pointed out that despite a shared bacterial diversity of 32% between microalgae feedstock and anaerobic digester samples, the resulting communities established in the microalgae digester were significantly different from the influent. Consequently, the influence of diversity-rich feedstock, especially in presence of anaerobic microorganisms (like it occurs in the primary sludge), over anaerobic digestion communities should be carefully explored in bioreactor configurations such as the AnMBR. In this system, the use of ultrafiltration membranes enhances the retention of niche and biofouling-related microorganisms (Robles et al., 2018; Skouteris et al., 2012). Furthermore, high solids retention capacity enhances microbial persistence resulting in microbial communities with high diversity and richness, according to 16S rRNA/rRNA gene sequencing results (Mansfeldt et al., 2019).

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

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

The second component explains 27% of the variability between samples and specially remarks a change in the structure of co-digester samples (Figure 1).

As shown in Figure 1, samples were categorized according to the digester and the feedstock. For the first categorical variable, two clusters were revealed by Adonis test (digester, $p<0.001$). Hence, the microbial structure of the co-digester and its reservoir was consistent in between but differed from the microbial structure of the digester samples. The second categorical variable used in the Adonis test revealed the existence of three clusters (feedstock, $p<0.011$), although four feedstock scenarios were analyzed in the present study. Thus, the differences in the microbial community structures of both AnMBRs should be attributed to the addition or not of a co-substrate and not to the species of microalgae fed to the reactor. In fact, microbial structure in the digester did not shift significantly when feeding *Scenedesmus* or *Chlorella*. The change in the microalgae did not either disturb the microbial structure of the co-digester, since the co-digester early stages samples are grouped with the *Scenedesmus* and primary sludge scenario samples (see top left corner samples in Figure 1). Finally, the differences among the digester samples were related to the effect of the SRT over the microbial population and the acclimation trend of the biomass, as previously mentioned.

The proximity between the samples collected when digesting *Scenedesmus* or *Chlorella* observed through the PCoA (Figure 1) suggest the potential use of the same anaerobic biomass to degrade these two algae. This is a remarkable fact and highlights the potential use of this acclimated biomass in microalgae-based bioenergy recovery processes. This concept which is based on a circular economy requires low-cost stages of microalgae disruption. An attractive strategy is to use these acclimated microbial communities as hydrolytic biomass sources and convert microalgae into biomethane through anaerobic digestion. Both Chlorophyta belonging genera are commonly found in fresh water and spontaneously grow over sewage-treated effluents (Garrido-Cardenas et al., 2018). Hence, the findings here reported support the use of

this biological strategy in a loop-system combining microalgae cultivation using anaerobically treated sewage-effluents, biomass harvesting and their further conversion into energy.

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

During SRT acclimation from 50 days up to 100 days in the digester AnMBR, slow-growing hydrolytic microorganisms were selected allowing the degradation of raw microalgae with remarkable methane yields (Table 2). Figure 2 shows the relative abundances calculated at phylum level from the $OTU_{0.97}$ compositions among samples of the AnMBR digester. The changes in the patterns reveals the effect of SRT over microbial composition.

During the operation at the lowest SRT (50 days) the dominant phyla observed were: 23.5% Chloroflexi, 16.6% Proteobacteria, 11.1% Planctomycetes and 9.7% Firmicutes (Table S4, samples xx). These four groups were present during the whole experimental period and are common groups of anaerobic digesters, as shown in different studies of full-scale systems (Calusinska et al., 2018; De Vrieze et al., 2018) and also in microalgae digesters (Córdova et al., 2018; Greses et al., 2018, 2017; Klassen et al., 2017; Sanz et al., 2017) or co-digesters (Li et al., 2017). However, their relative abundances changed under different SRT operation as other microbial groups like Bacteroidetes, Cloacimonetes, Spirochaetes, Aminicenantes and Candidatus Dojkabacteria (WS6 phylum) thrived in the system and co-existed with the previous phyla. The operation at 70 days SRT with an HRT of 50 days was characterized by the remarkable presence of the Ca. Dojkabacteria (14.8%, see Table S4). This novel group is poorly described and none of the belonging members has been isolated yet. Their early identification by Dojka et al. (2000) using culture-independent approaches (16S rRNA gene cloning) suggested their importance in organic-rich environmental anaerobic niches. Up to the present date and to the knowledge of the authors of this manuscript, no other studies have clearly described their function in anaerobic digesters for microalgae conversion into energy. Interestingly, Qiao et al. (2013) observed Ca. Dojkabacteria during anaerobic digestion of corn

straw. According to more recent metagenomic findings Ca. Dojkabacteria related OTUs have xylan disruption capacity (Solden et al., 2016). This sugar is commonly observed in Chlorophyta cell walls (Baudelet et al., 2017; Domozych, 2014) and hence, the role of this phylum in microalgae degradation could be suggested from these findings.

However, Ca. Dojkabacteria presence decreased in the AnMBR after changing the HRT from 50 to 15 days. An antagonist response was observed for Firmicutes phylum, which was favoured during increased SRT operation at 70 days, reaching relative abundance values up to 12.5% (sample 211, Figure 2). Different members of Firmicutes are commonly reported in complex polysaccharide anaerobic degradation, since they can release enzymes to the environment and disrupt complex molecules (Calusinska et al., 2018; Cheng et al., 2014). The Firmicutes phylum decreased in terms of relative abundance after the HRT reduction from 50 to 15 days, suggesting that other microbial groups have higher affinity for the substrates and thrived in the AnMBR. Despite the maintenance of a SRT of 70 days, the reduction of the HRT increases the organic loading rate of the system and reduces the contact time in between the soluble phase and the microorganisms. Thus, lower HRT can affect the mass transference of the system and enhance microbial groups with lower specific rates of substrate utilization.

During operation at high 15 days HRT and high SRT the relative abundances of Bacteroidetes and Aminicenantes phyla increased at 100 days SRT. Both groups remained in the AnMBR digester during operation at 70 days SRT, although their relative abundance values were lower over time and especially at the end of *Scenedesmus* digestion (samples 483 and 624, Figure 2). Then, for Chlorella digestion also at high SRT of 100 days and low HRT of 15 days, changes in the phyla profiles were observed. Consequently, the relative abundances of both Bacteroidetes and Aminicenantes were lower for Chlorella digestion scenario than for Scenedesmus. Both phyla have been related to the core of wastewater anaerobic digestion systems in a recent study performed over twenty years targeting the 16S rRNA gene

(Calusinska et al., 2018). The role of Bacteroidetes in the present work could be more heterogeneous, as different members related to this phylum are involved in both polysaccharide and peptide degradation. Indeed, Bacteroidetes has been remarked as a key phylum continuous raw microalgae digestion for methane production (Klassen et al., 2017). On the other hand, Farag et al. (2014) early suggested the wide potential metabolic implication of Aminicenantes in anaerobic environments. However, little is known about this group as none of the representative members of this has been isolated yet in a pure culture, but recent findings suggest their importance in hydrogen and acetate production after saccharolytic degradation (Kadnikov et al., 2019). Hence, they could play an important role during microalgae degradation at high SRT as methanogenic substrate donors.

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

3.4. *A microbial core with similar biomethanization pathways from microalgae and primary sludge*

In the present work another AnMBR was run in parallel using the same microalgae biomass plus primary sludge collected from the WRRF primary settler. Similar communities might be established when treating the same feedstock sources, as a result of the stabilization of a microbial core in biogas reactors (Zuopeng et al., 2019). In fact, a microbial core for microalgae biomethanization was elucidated in this study, as the Venn diagram shows (Figure 3). A total number of 578 $OTU_{0.97}$ were shared between the AnMBR operated under the different scenarios.

Also, the venn diagram revealed the presence of unique $OTU_{0.97}$ in the four scenarios. The scenario with the highest number of unique members was Chlorella and Primary Sludge (131 $OTU_{0.97}$). The remaining scenarios had 103, 109 and 92 specific $OTU_{0.97}$ (Scenedesmus, Scenedesmus and Primary Sludge and Chlorella, respectively) (Figure 3). The small difference between the digestion and co-digestion scenario for Scenedesmus contrasts with the high difference in terms of unique $OTU_{0.97}$ of Chlorella digestion and co-digestion, which showed the lowest and highest value of unique members. These findings are similar to the alpha diversity analysis results, since Scenedesmus scenarios had higher specificity than Chlorella scenarios. However, the unique $OTU_{0.97}$ were not presence in relative abundance over 0.7% in any sample. Hence, the presence of specific members in each different scenario might not be as important as the persistence of a microbial core of 578 $OTU_{0.97}$ that are shared in between the four scenarios.

The most abundant $OTU_{0.97}$ found in the microbial core were related to uncultured members of Anaerolineaceae family (phylum Chloroflexi), Synergistaceae (Synergistetes) and the candidate phylum Cloacamonas; besides Smithella and Methanosaeta (Figure 4). *Smithella* genus (order Syntrophobacterales) was predominantly observed in the co-digester, coinciding its highest values within the highest presence detected of *Methanosaeta* (order Methanosarcinales). As reported by Leng et al. (2018), both genera are commonly found in anaerobic digestion processes and play an important role during methane production after fatty acid conversion into a more reduced form *i.e.* acetate. Although no other omics approach rather than DNA amplicon sequencing was performed in this work, the findings of both microbial members suggest that methane was mainly produced by *Methanosaeta* through the *Smithella* pathway. Indeed, the comparison of the consensus sequence for each $OTU_{0.97}$ reported a 99% and 96% identity with *Methanosaeta concilii* and *Smithella propionica*. This would have been promoted through the addition of the co-substrate to balance the C:N ratio of the influent. This

411 strategy can also have a positive impact on enzymatic synthesis stimulation improving
412 microalgae anaerobic digestion yields (Sialve et al., 2009) and consequently, methanogenic
413 population. In contrast, the microalgae digester presented a lower abundance of *Methanosaeta*
414 (1.3% when digesting *Scenedesmus* and 0.6% with *Chlorella*) and *Smithella* was detected at
415 very low values (4.8% and 0.6%, respectively). This could be related to the less balanced
416 scenario for methane production as the C:N ratio in the digester was lower than in the co-
417 digester. In fact, as shown in Figure 4 higher presence of syntrophic members classified under
418 the Syntrophobacterales family were observed for the two co-digestion scenarios.
419 The still poorly characterized phylum Chloroflexi showed up a very high presence in all
420 samples. Summarizing, three $OTU_{0.97}$ were observed in the digester when the co-substrate was
421 added as well as when only microalgae was digested. However, more relative abundance of
422 Anaerolineaceae clusters I and II were observed in the co-digestion scenarios, compared to the
423 digestion scenarios (Figure 4). According to the review from McIlroy et al. (2017) all isolated
424 members of this family are donors of acetate after fermentation of carbohydrates. Also, this
425 family has been proposed as biological disrupters of microalgae (Greses et al., 2017; Sanz et
426 al., 2017) besides macroalgae (Zou et al., 2018) and would be involved in the production of
427 other fermentation products such as lactate, hydrogen and formate. Interestingly, the cells of
428 the microorganisms belonging to this group are filamentous type. A recent study from Bovio
429 et al. (2019) supports their importance in granule generation in Up-flow Anaerobic Sludge
430 Blanket (UASB) systems. This is a key capacity also during biofouling and cake formation
431 processes in AnMBR systems that could explain the dominance of Anaerolineaceae in the
432 present study. Moreover, as reported by McIlroy et al. (2017), Anaerolineaceae and
433 *Methanosaeta* are commonly associated forming a complex filamentous network. If this group
434 was major donors of acetate to *Methanosaeta* in this work, the association in a “spaghetti-like”

structure of Anaerolineaceae and the aceticlastic methanogen could have promoted the metabolites transfer flux between both groups, resulting in high methane production rates.

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

This microbial core has been defined in terms of dominant relative abundances (see Tables S1-S4 for further details). However, further research is needed in order to develop future methodologies for monitoring the dynamics of these groups in anaerobic systems producing energy from microalgae. Since microalgae degradation is complex from a metabolic perspective due to the heterogeneous composition of microalgae cell walls (Baudelet et al., 2017), targeting the members of the microalgae-degrading microbial core could be an effective strategy to monitor microalgae digesters. A future necessary step would be the design of specific probes or oligonucleotides that can target the rRNA and provide the activity levels of these groups. Besides, qPCR approaches or 16S rRNA gene sequencing coupled to flow-cytometry sorting systems (Rinke, 2018; Wang et al., 2010) or including a spike control (Stämmler et al., 2016) could provide absolute measurements of these relevant microorganisms for bioenergy production. Towards the development of future microbial-based models of anaerobic digestion of complex feedstocks that are produced in WRRF this effort should be considered, since microbial communities cannot be longer overstated (Widder et al., 2016).

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

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

Figure 5 shows the results from the fitted PLS-DA regression model. As can be seen in this figure the samples from the microalgae digestion are well separated from the samples from co-digestion. To elucidate the most discriminant groups between both digestion substrates, the variable importance in the projection (VIP) was calculated. The first 30 microbial members sorted by the highest VIP value retrieved from PLS-DA are shown in Figure S2. Genera belonging to Actinobacteria, Atribacteria, Chloroflexi, Cloacimonetes, Firmicutes, Proteobacteria, Spirochaetae, Verrucomicrobia (Bacteria) and WSA2 (Archaea) were among the most discriminant ones. Some of them are classified inside of the dominant phyla observed in both AnMBRs (Figure 2). However, others like *Ca. Caldatribacterium* (phylum Atribacteria) are detected at very low abundances (1-2%) but were highly discriminating between samples. According to Dodsworth et al. (2014) this group is able to perform saccharolytic fermentation from cellulosic as well as hemi-cellulosic substrates. Since cellulose is present in common WTTP primary sludge stream in about 30-50% of the influent suspended solids (Crutchik et al., 2018), the thrive of this bacteria group during co-digestion but not when only microalgae was digested could be related to the higher presence of this complex polysaccharide in the feedstock. *Treponema*, a Spirochaetae member, was also among the most discriminant and found only in the samples from co-digestion. The presence of this group was

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

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

The use of microbial-rich biomass sources as co-substrate might present a drawback when using biological strategies to convert microalgae into biogas. The primary sludge strongly shaped the microbial communities in the co-digester as shown in the PCoA (Figure 1). From a microbial ecology perspective, this could also be partially related to the accumulation of co-substrate incoming microorganisms and groups entering the system might be viable during microalgae co-digestion. Primary sludge has a high species richness. Although its diversity has not been evaluated on its own in the present study and is rarely evaluated in similar studies, Ju et al. (2017) observed 3424 $OTU_{0.97}$ in the primary sludge seed used for their anaerobic digestion trials. However, this study only relied on the microbial characterization through the biomarker 16S rRNA gene and could not therefore evaluate the survival of these potential microbial groups present in the influent. Further research using transcriptomic approaches might help to elucidate the activity levels of the microorganisms observed. Since some of microorganisms are anaerobic and might be acclimated to cellulolytic components present in the primary sludge, they could improve the later digestion of microalgae during the co-digestion.

The present work has demonstrated that a core representing the 57% of the microbial diversity is maintained over time in bioreactors treating microalgae. The maintenance of a core microbiome in anaerobic reactors was reported to be extremely relevant in order to maintain

513 the functional status (Rivière et al., 2009). Peces et al. (2018) reported a convergent diversity
514 after 120 days of continuous operation of four different anaerobic digesters, inoculated with
515 different sources but identically operated to produce biogas from a cellulose:casein feedstock.
516 According to these authors, the microbial core contained a 78% of the anaerobic digesters
517 diversity. The neutral theory predicts that populations are driven by deterministic factors such
518 as SRT, HRT and OLR, as it has been demonstrated using different inocula to anaerobically
519 degrade cellulose (Vanwonterghem et al., 2014). Up to date, most of the microbial core focused
520 studies have only used the target 16S rRNA gene. Therefore, further research is needed in order
521 to elucidate the active microbial core, as minor groups might have a relevant role during
522 microalgae digestion. This has been suggested in the present study through application of PLS-
523 DA that remarks the importance of the presence or absence of certain groups to shape microbial
524 structures, despite of their low relative abundances. On this basis, RNA-based sequencing (De
525 Vrieze et al., 2018) could facilitate a better profile of key microorganisms during microalgae
526 digestion and especially during co-digestion. Functional profiling of anaerobic communities is
527 a necessary step towards the development of new probes to monitor the wealth of anaerobic
528 digesters from a microbiologist perspective. Also, to retrieve more accurate information in
529 future microbial ecology studies of anaerobic digesters, efforts in targeting the active cells like
530 active cell sorting in flow cytometers and later sequencing (Nakamura et al., 2016) or RNA-
531 based sequencing (De Vrieze et al., 2017) would be required.

532 **Conclusions**

533 A microbial core has been elucidated in this study from four different scenarios for raw
534 microalgae conversion into biogas. The high presence of several Anaerolineaceae members
535 highlights the importance of saccharolytic and peptidic hydrolysis and fermentation. The
536 dominance of *Smithella* and *Methanosaeta* suggest the relevant role of syntrophic and
537 methanogenic pathways for bioenergy production from raw microalgae. This association was

more important during co-digestion than when only microalgae was digested, probably because of the composition of primary sludge. Nonetheless, no significant change in the acclimated communities was observed during microalgae shift from *Scenedesmus* to *Chlorella*. Instead, the microbial core was maintained over time in both AnMBR.

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Authors contributions

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

Supplementary data

Supplementary data associated to the present study can be found in the digital version of this manuscript

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