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Additional Information

Microbial community characterization during anaerobic digestion of *Scenedesmus* spp. under mesophilic and thermophilic conditions

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Abstract

Microbial communities were thoroughly characterized in a mesophilic anaerobic membrane bioreactor (AnMBR) and a thermophilic continuous stirred tank reactor (CSTR), which were both treating recalcitrant microalgal biomass dominated by *Scenedesmus*. 16S rRNA amplicon sequencing analysis was performed when the AnMBR achieved 70% algal biodegradation and revealed high microbial diversity, probably due to the high solid retention time (SRT) of the AnMBR configuration. The bacterial community consisted of Chloroflexi (27.9%), WWE1 (19.0%) and Proteobacteria (15.4%) as the major phyla, followed by Spirochaetes (7.7%), Bacteroidetes

(5.9%) and Firmicutes (3.6%). These phyla are known to exhibit proteolytic and cellulolytic capabilities required to degrade the *Scenedesmus* cell-wall. *Methanosaeta* was the most abundant methanogen detected in the AnMBR suggesting that methane was mainly produced by the acetoclastic pathway. In comparison, the thermophilic CSTR achieved 32.6% algal biodegradation, and its bacterial community had fewer Operational Taxonomic Units (977 OTUs) than the AnMBR (1396 OTUs), as is generally observed for high temperature biogas reactors. However, phyla with high hydrolytic potential were detected such as Firmicutes (34.6%) and the candidate taxon EM3 (38.7%) in the thermophilic CSTR. Although the functional metabolism of EM3 in anaerobic digesters is unknown, the high abundance of EM3 suggests that this taxon plays an important role in the thermophilic, anaerobic degradation of *Scenedesmus*. The abundant syntrophic bacteria and the detection of hydrogenotrophic methanogens in the thermophilic CSTR suggest that the hydrogenotrophic pathway was the dominant pathway for methane production in this reactor.

Keywords

Anaerobic digestion; membrane technology; microbial community analysis; *Scenedesmus* spp.

1. INTRODUCTION

Dwindling fossil fuel reserves and the notable environmental problems associated with their use have created an urgent need to develop renewable fuels. Biogas production through anaerobic digestion is a promising renewable energy technology that has gained international attention [1]. Feedstocks such as food waste or lignocellulosic biomass [2,3] have been studied as substrates for anaerobic digestion. One recent development is the use of microalgae, both as a way to recover nutrients from wastewater effluents and as a feedstock for biogas production through anaerobic

digestion [4] since previous studies reported that microalgae biomethanization is an economically feasible approach when their cell lipid content is lower than 40% [5]. Microalgae exhibit several characteristics that make them a promising biomass resource such as a high photosynthetic efficiency, a growth rate of 20-30 fold higher than energy crops and no direct competition with food production since microalgae could be grown on non-arable land [6]. However, the use of wastewater streams to cultivate microalgal biomass typically results in the growth of specific strains such as *Scenedesmus* [7], which possesses a rigid cell-wall. This cell-wall is composed of cellulose, hemicellulose and lignin [6,8] and is highly resistant to biological degradation [9].

Several studies have shown enhancement of microalgae biodegradability by applying different pretreatments for disruption of the algal cell-wall [9]. As an alternative to pretreatment, Ras et al. [10] found that biogas production from anaerobic digestion of microalgae was enhanced when the reactor's solid retention time (SRT) was increased. High SRT could promote the retention of microorganisms that degrade recalcitrant microalgal biomass and thus increase the hydrolytic potential of the anaerobic process. Nevertheless, anaerobic digestion at high SRT performed within a conventional continuous stirred tank reactor (CSTR) requires an increase of reactor working volume needed for a given treatment flow. This drawback can be overcome by using advanced reactor configurations such as an anaerobic membrane bioreactor (AnMBR), which allows decoupling the SRT and the hydraulic retention time (HRT). Although an AnMBR configuration increases the reactor complexity and requires a higher initial economic investment, this kind of reactor is an advantageous alternative since it can be operated at long SRT, with a high treatment flow and with a reduced surface requirement.

Alternatively, hydrolytic activity can be increased by carrying out the anaerobic digestion under thermophilic conditions since thermophilic microbial populations exhibit higher enzymatic activity [11], although the thermophilic process is more prone to instability than the mesophilic one due to inhibition by free ammonia [12], especially when substrates with high protein content such as

microalgae are digested [13].

Microbial communities involved in anaerobic digestion processes are highly variable in their composition, which is determined by factors like inoculum source, operational conditions such as temperature, SRT or Organic Loading Rate (OLR), and the type of feedstock used [14]. In this context, little is known about the bacterial consortium capable of degrading *Scenedesmus* under anaerobic conditions, and this lack of knowledge limits optimization of the process. Only a few studies have focused on the archaeal [15] and bacterial communities [14,16] involved in the anaerobic digestion of different strains of microalgae. However, previous studies have not been described the microorganisms involved in the mesophilic anaerobic digestion within an AnMBR of *Scenedesmus* grown on wastewater effluent, nor have there been reports of the thermophilic anaerobic digestion within a CSTR of *Scenedesmus* in the absence of free ammonia inhibition. Hydrolysis is usually the rate limiting step during anaerobic digestion of the polymeric components of organic matter [9]. Thus, thorough characterization of the microbial populations involved would provide valuable information.

Accordingly, the aim of the present study was to identify the bacterial and archaeal taxa involved in the anaerobic digestion of recalcitrant *Scenedesmus* biomass to produce biogas. To achieve this aim, high-throughput 16S rRNA amplicon sequencing was used to characterise the microbial communities in a mesophilic anaerobic membrane bioreactor (AnMBR) and a thermophilic continuous stirred tank reactor (CSTR). The feedstock of both reactors was *Scenedesmus*, and we performed microbial community analysis once the reactors had operated at steady state for a period of 3 months.

2. MATERIAL AND METHODS

2.1. Microalgal feedstock

As a bioremediation technique, microalgae was cultivated in a Membrane PhotoBioReactor pilot plant (MPBR) in order to remove nutrients from the effluent of an AnMBR pilot plant, as

previously described by Viruela et al. [7]. This microalgal biomass, mainly composed of *Scenedesmus* spp. [7], was concentrated by filtration before being fed to two laboratory-scale reactors, a mesophilic AnMBR and a thermophilic CSTR, according to the OLR described for each reactor in the next sections. *Scenedesmus* biomass was concentrated by filtration using an external cross-flow, ultrafiltration hollow fibre membrane system (HF 5.0-43-PM500, PURON® Koch Membrane Systems), which has a surface area of 2.1 m² and a nominal pore size of 500 kDa MWCO (Molecular Weight Cut-Off). In this system, microalgae from MPBR were pumped through the membrane, generating a flow rate that allows the filtration to take place. The filtration proceeded until the concentration of microalgae (as determined by COD – Chemical Oxygen Demand) had increased from an initial COD of 500 mgO₂·L⁻¹ to the desired COD (Table 1), and thus obtaining the algal biomass batch.

The batches of microalgal feedstock for every reactor were stored at 4°C until it was consumed and then new batches were prepared for a total of 7 batches that were ultimately fed to the reactors. The microbial community was analysed during a period in which the reactors were fed from the same initial microalgae in order to avoid a batch effect. The composition of the microalgal feedstock is shown in Table 1.

Table 1. The microalgal influent composition in both reactors as mean ± standard deviation ¹.

Parameter	Unit	Thermophilic CSTR	Mesophilic AnMBR
		Influent	Influent
TSS	mg TSS·L ⁻¹	7169 ±55	3982 ±77
COD _{Total}	mg O ₂ ·L ⁻¹	9913 ±115	6033 ±137
VFA	mg HAc·L ⁻¹	173.5 ±10.8	187.8 ±14.2
ALK	mg CaCO ₃ ·L ⁻¹	401 ±11	407 ±8
NH ₄ -N	mg N·L ⁻¹	44.5 ±7.1	48.0 ±2.5
N _{Total}	mg N·L ⁻¹	622.0 ±30.0	375.9 ±53

¹ The OLR for both reactors was established by adjusting the microalgal feedstock concentration based on its COD.

2.2. Anaerobic reactors description and operation

Scenedesmus spp. was digested anaerobically in two lab-scale reactors (a mesophilic AnMBR and a thermophilic CSTR) under different operational conditions. Both reactors were equipped with sensors for continuous monitoring of pH, temperature, oxidation reduction potential and pressure, all of which were recorded by a custom data logging script written in visual basic. Reactor temperature was maintained with a water jacket connected to a temperature-controlled water bath. Biogas volume was measured with a gas flow meter (μ Flow, Bioprocess Control. Lund, Sweden). Reactors were fed once a day using fresh microalgal biomass, and collected effluent was analysed weekly to control the digestion process.

2.2.1. Mesophilic AnMBR

Mesophilic digestion of microalgae was performed at 35 °C in a 12.4 L lab-scale AnMBR with a 9.9 L working volume. The reactor was mixed by recirculation of biogas from the headspace to the bottom of the reactor as well as to the membrane module in order to control biofilm formation. Likewise, the sludge was maintained homogenised by its continuous recirculation from the reactor tank to the membrane module. The surface area of the hollow-fibre ultrafiltration membrane module (PUR-ON[®] Koch Membrane Systems) was 0.42 m² with a nominal pore size of 0.05 μ m.

The reactor was operated for 3 years and was inoculated with 9.9 L (the entire working volume) of sludge from a conventional mesophilic anaerobic digester located at Carraixet WWTP (Valencia, Spain), which digests primary and secondary sludge produced from wastewater treatment. The start-up of the laboratory reactor was carried out in a CSTR configuration and, after 150 days of operation, the configuration was modified by adding an ultrafiltration membrane module to decouple the SRT and the hydraulic retention time (HRT). Thereafter, this AnMBR configuration allowed operation at high SRT with a high treatment flow rate. Herein, as a part of a large

multifarious study, we report an observation period of 160 days where SRT was set at 100 days by removing $99 \text{ mL}\cdot\text{d}^{-1}$ of anaerobic slurry from the reactor, and HRT was at 15 days with an OLR of $0.4 \text{ g}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$. Under these operational conditions the process exhibited stability during the last 90 days, at which time samples for microbial population analysis were taken during 3 consecutive weeks.

2.2.2. *Thermophilic CSTR*

Thermophilic digestion of microalgal biomass was performed for 2 years at $55 \text{ }^\circ\text{C}$ in a 2 L CSTR with a 1.6 L working volume. The reactor was mixed with mechanical stirring and was inoculated from a thermophilic, pilot-scale digester located at Valladolid (Spain) which digests primary and secondary sludge produced from wastewater treatment. In the present study, results are reported from an experimental observation period of 160 days during which time the SRT was set to 35 days and the OLR was set to $0.3 \text{ g}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$. Process stability was observed during the last 90 days under this configuration, during which time the samples for microbial community analysis were collected.

2.3. Analytical Methods

Reactor samples were regularly analysed in order to monitor the biological process. Once a week, parameters such as nitrogen (4500-N-C followed by 4500- $\text{NO}_3^- \text{ H}$), ammonium (4500- $\text{NH}_3\text{-G}$), phosphorus (4500-P-B followed by 4500-P-F), and phosphate (4500-P-F) concentrations as well as total and soluble COD (5220-B and 5220-C, respectively) and total and volatile suspended solids (2540-D and 2540-E, respectively) were measured according to Standard Methods [17] whose identifiers are given in parenthesis.

Once ammonium was measured along with pH and temperature, the NH_3 concentration in the reactors was calculated using the equilibrium equation (Eq. 1) proposed by Zhang et al. [18] in which TAN is the total ammonia nitrogen in the reactor and temperature (T) was expressed in Kelvin.

$$[\text{NH}_3] = \frac{\text{TAN}}{1 + \frac{10^{-\text{pH}}}{10^{-(0.09018 + 2729.92/T)}}} \quad [\text{Eq. 1}]$$

Volatile Fatty Acids (VFA) and alkalinity were measured by titration using the method proposed by Moosbrugger et al. [19]. The methane fraction in biogas was measured three times a week using a Gas Chromatograph fitted with a Flame Ionization Detector (GC-FID, Thermo Scientific). For this purpose, a volume of 0.5 mL of biogas was sampled from the headspace of the reactor through a septa by gas-tight syringe, and then injected into a 15 m x 0.53 mm x 1 μm TRACER column (Thermo Fisher), which was maintained at 40°C. Helium was used as carrier gas with a flow rate of 5 mL·min⁻¹ and the calibration standard was pure methane (99.99%, Air Products Inc.).

All the analyses carried out for every point were performed in triplicate in order to calculate the average and the standard deviation shown in tables and graphs.

2.4. Microbial population analysis

Once steady-state was reached, which is defined as the time period when the parameters of the process remain constant, the microbial communities were analysed in both reactors as well as in the microalgal feedstock. For this purpose, a total of 5 samples were taken from each reactor at weekly intervals during the steady-state and 4 samples were analysed from the feedstock during the same period. The samples from the mesophilic AnMBR were taken at 128, 134, 142, 148 and 156 days, and samples from the thermophilic CSTR were collected at 126, 133, 139, 147 and 155 days. Though samples of the microalgal feedstock were analysed during that same period (at 127, 136, 142 and 155 days), they were derived from the same batch, and therefore these samples are technical replicates. All samples were immediately frozen at -20 °C after collection. Genomic DNA was extracted from 1 mL of sample which was centrifuged at 10000 rcf for 5 min to remove the liquid, and then the pellet was resuspended in 300 μL of S.T.A.R. buffer (Roche) to stabilize the nucleic acids in the sample. Cell disruption was carried out by adding 0.25 g of acid-washed glass

beads and bead-beating twice in a MagNaLyser machine at 6500 rpm for 20 seconds each time, followed by centrifugation at 13000 rcf for 5 min to enable the DNA recovery in the supernatant. DNA was then extracted using the MagMidi kit (LGC Genomics) for the KingFisher Flex robot according to the manufacturer's protocol. DNA concentration was measured by Qubit fluorometer with Quant-iT dsDNA Br assay kit (Invitrogen), and the DNA quality was assessed with the Nanodrop ND 1000 spectrophotometer (Thermo Scientific).

DNA extracted from the samples was amplified with the primers Pro341F (5'-CCTACGGGNBGCASCAG-3') and Pro805R (5'-GACTACNVGGGTATCTAATCC-3') [20], which target the V3-V4 hypervariable regions of the 16S rRNA gene for both bacteria and archaea with an expected amplicon size of 465 bp. The polymerase chain reaction (PCR) mixture (25 µL) consisted of 2.5 µL of DNA (5 ng/µL), 12.5 µL of iProof HF Master Mix (BIO-RAD, USA), 5 µL of each primer (1 µM). The PCR cycling consisted of an initial denaturation step at 98°C for 3 min followed by 30 cycles consisting of 98°C for 10 s, 55°C for 30 s, and 72°C for 30 s, and with a final elongation step at 72°C for 5 min. PCR products were verified by electrophoresis on a 1% agarose gel. The PCR-amplified samples were barcoded using the Nextera XT DNA Library Preparation Kit (Illumina) according to manufacturer's protocol. The concentration of barcoded amplicons was determined using a Qubit fluorometer with Quant-iT dsDNA BR assay kit (Invitrogen) and each amplicon was adjusted to equimolar concentration according to the Illumina protocol for 16S Metagenomic Sequencing Library Preparation. The library was sequenced on an Illumina MiSeq machine with MiSeq reagent kit v3 (600-cycle), according to the manufacturer's protocol. Sequence data from the samples were analysed with a suite of programs as follows: First, the paired-end reads from each sample were merged using the program PEAR [21]. Then the sequences were quality filtered using PRINSEQ [22] by requiring a mean quality score of 30 and a minimum length of 350bp. Primer sequences were removed using the *trim.seqs* function of the program Mothur [23].

Chimeric sequences were removed followed by clustering into operational taxonomic units (OTUs) at 97% sequence identity (OTU_{0.97}) by USEARCH [24,25] as implemented in the Quantitative Insights Into Microbial Ecology (QIIME) 1.9.1 software package [26] using the Greengenes database *gg_13_8* [27]. After raw sequences were processed, a final total number of sequences of 352832 were obtained with an average of 24111 (\pm 3978) read counts for each sample. A rarefaction lower limit of 16500 sequences per sample was applied for diversity analysis along with estimation of Simpson, Shannon, Gini indices and the number of OTUs observed, which were used to obtain both richness and evenness in the samples. A similarity matrix of weighted UniFrac distances was used for ordination by principal coordinate analysis (PCoA) in order to determine changes in the community composition as a consequence of the anaerobic treatment of the microalgae under mesophilic and thermophilic conditions. Likewise, principal components analysis (PCA), based on the measured parameters in the reactors, was performed using the software PAST [28] in order to show the influence of the reactor environments. PAST [28] was also used to perform the ANOSIM statistical analysis with a p-value of 0.05 in order to test for differences in microbial community composition between the reactors and the feedstock. The sequence data for the 14 samples analysed in this work was uploaded to the Sequence Read Archive (SRA) as accessions SRR5436370 to SRR5436383 as part of BioProject PRJNA324836.

3. RESULTS AND DISCUSSION

3.1. Anaerobic digestion

In the present study, two lab-scale anaerobic reactors (a mesophilic AnMBR and a thermophilic CSTR), were fed with microalgal feedstock dominated by *Scenedesmus* spp. in a study which aimed to optimize the anaerobic biodegradation of the microalgal biomass. Thus, the process efficiency was evaluated by monitoring different parameters such as COD removal, total suspended solids (TSS), ammonium/free ammonia concentrations and biogas production per litre of reactor working volume (Figure 1).

The mesophilic AnMBR reached a COD removal of 70% (Figure 1a), which corresponds to an average methane yield of $242.1 \text{ mL CH}_4 \cdot \text{g COD}_{\text{influent}}^{-1}$. The solids concentration was reduced from a maximum value of $9070 \text{ mg} \cdot \text{L}^{-1}$ at the beginning of the experimental period to $7263 \text{ mg} \cdot \text{L}^{-1}$ when steady-state was reached (Figure 1b) as a consequence of the high hydrolytic activity of the anaerobic microorganisms involved in the process, which increased biogas production from 109.3 to $158.7 \text{ mL} \cdot \text{L}_{\text{reactor}}^{-1} \cdot \text{d}^{-1}$ (Figure 1d, Table 2). Ammonium concentrations stabilized around $284.1 \text{ mg NH}_4\text{-N} \cdot \text{L}^{-1}$ and free ammonia remained lower than typical inhibition concentrations ($80 \text{ mg NH}_3\text{-N} \cdot \text{L}^{-1}$) (Figure 1c) reported by Garcia and Angenent [29] owing to the combined effect of both, a low ammonia concentration and pH value, since pH stabilized around 7.03 in our reactor while Garcia and Angenent [29] maintained their reactor pH around 7.60. This result is in accordance with Ras et al. [10], who demonstrated that mesophilic digestion of *Chlorella vulgaris* microalgae within a CSTR achieved 51% COD removal when the SRT increased from 16 to 28 days, although this microalgal strain exhibits lower resistance to biological degradation than *Scenedesmus* [30]. These results show that anaerobic digestion of microalgae at high SRT of 100 days by using membrane technology can achieve high biogas yields without requiring costly pretreatments.

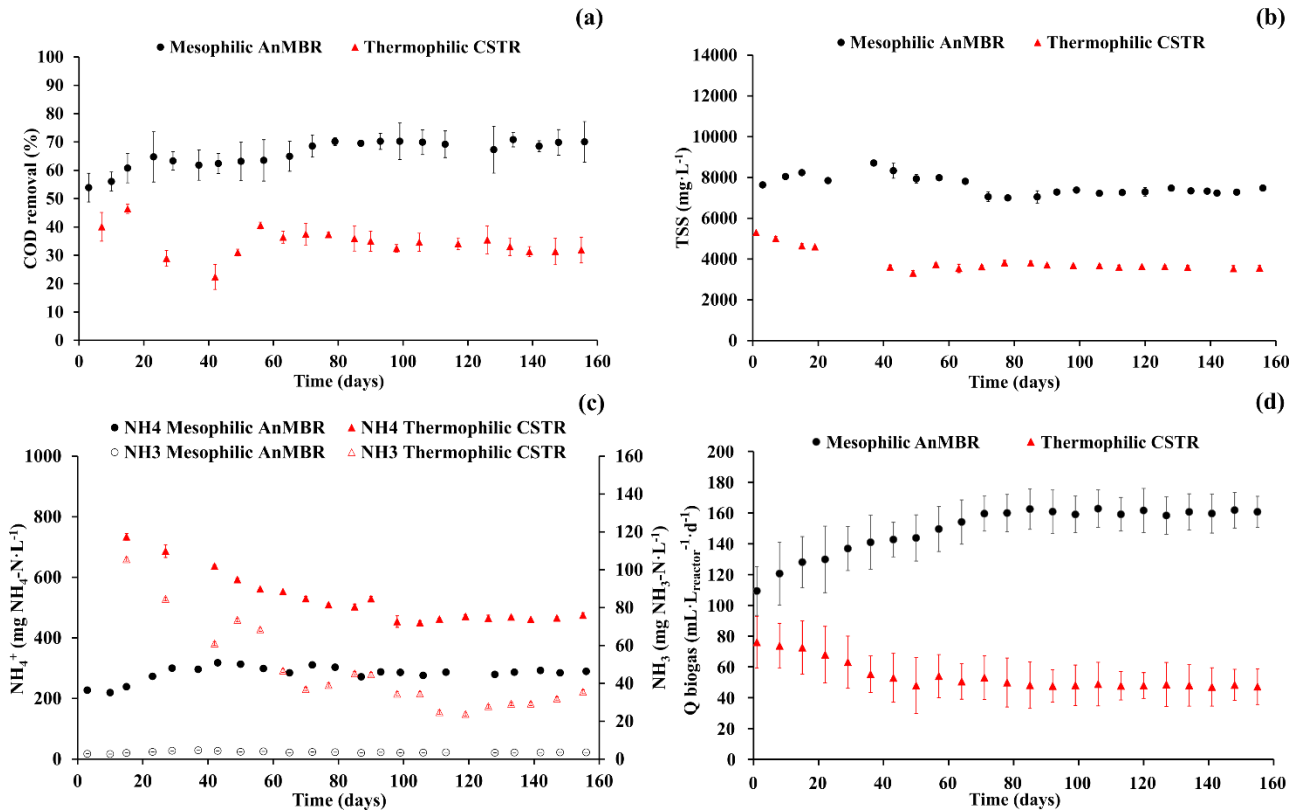


Figure 1. Percentage of COD removal (a) and concentration of TSS (b), NH_4^+ and NH_3 (c) and biogas production (d) during progression of the anaerobic digestion of *Scenedesmus* under mesophilic and thermophilic conditions. Error bars represent the standard deviation (SD) of replicate measurements.

Anaerobic digestion of microalgae under thermophilic conditions in the CSTR resulted in a stable COD removal of 32.6% (Figure 1a) and a biogas production of $48.5 \text{ mL} \cdot \text{L}_{\text{reactor}}^{-1} \cdot \text{d}^{-1}$ (Figure 1d, Table 2), corresponding to a methane yield of $98.4 \text{ mL CH}_4 \cdot \text{g COD}_{\text{influent}}^{-1}$ with a TSS concentration of $3700 \text{ mg} \cdot \text{L}^{-1}$ within the reactor (Figure 1b, Table 2). This lower biodegradability as compared to the mesophilic process was mainly due to the fact that the thermophilic CSTR was operated at a SRT of 35 days, which was 2.9-fold lower than the SRT set for the mesophilic AnMBR. Likewise, the COD removal achieved in the thermophilic anaerobic digestion of *Scenedesmus* was even lower than the results reported by Ras et al. [10] from mesophilic anaerobic digestion of *Chlorella vulgaris* at a 28 days SRT (51%), which demonstrates the high resistance of *Scenedesmus* to biological degradation and the need to adapt the process conditions to the microalgal strain.

Ammonium concentrations stabilized around $463.7 \text{ mg NH}_4\text{-N}\cdot\text{L}^{-1}$ (Figure 1c) which is related to the fact that HRT and SRT were not decoupled due to the absence of a separation system.

Nevertheless, free ammonia did not reach inhibitory concentrations [31], which was confirmed by the stable volatile fatty acids and high alkalinity in the effluent (see Table 2). The results obtained from both anaerobic reactors are summarized in Table 2.

Table 2. Mean and standard deviation of the process parameters measured during the stable period for the thermophilic CSTR and mesophilic AnMBR.

Parameter	Unit	Thermophilic CSTR	Mesophilic AnMBR
TSS	$\text{mg TSS}\cdot\text{L}^{-1}$	3700 ± 89	7263 ± 147
$\text{COD}_{\text{total}}$	$\text{mg O}_2\cdot\text{L}^{-1}$	6982 ± 47	11750 ± 157
VFA	$\text{mg HAc}\cdot\text{L}^{-1}$	267.5 ± 40.1	ND ²
ALK	$\text{mg CaCO}_3\cdot\text{L}^{-1}$	1620 ± 67	1495 ± 22
N-NH ₄	$\text{mg N}\cdot\text{L}^{-1}$	463.7 ± 7.8	284.1 ± 6.5
N-NH ₃	$\text{mg N}\cdot\text{L}^{-1}$	30.68 ± 2.71	3.46 ± 0.07
pH	-	7.38 ± 0.21	7.03 ± 0.13
$\text{COD}_{\text{removal}}$	%	32.6 ± 1.6	69.9 ± 0.7
Q_{biogas}	$\text{mL}\cdot\text{L}_{\text{reactor}}^{-1}\cdot\text{d}^{-1}$	48.5 ± 1.6	158.7 ± 1.4
CH ₄	%	60.8 ± 1.7	62.7 ± 1.3
Y^{CH_4}	$\text{mL}_{\text{CH}_4}\cdot\text{gCOD}_{\text{inf}}^{-1}$	98.4 ± 15.1	242.1 ± 11.0

² Values were lower than limit of detection.

3.2. Microbial community analysis

Microbial community composition of the microalgal anaerobic digestion was characterized once both reactors had reached steady-state. Although the microalgal feedstock is mostly composed of

Scenedesmus spp. biomass, it is in fact a mixed community of microbes. Thus its prokaryotic community composition was also characterized in order to compare it to the reactors and ensure that residual DNA from feedstock biomass is not skewing the apparent community composition in the reactors.

The diversity analysis showed that the number of OTU_{0.97} in the mesophilic reactor was higher than in the thermophilic reactor (Figure S1). This lower microbial diversity at higher temperature is in accordance with Vanwonterghem et al. [32], who detected a low microbial diversity when cellulose was anaerobically degraded under thermophilic digestion, and also has been reported previously by Li et al. [33], who found that temperature is one of the most influential factors in the distribution of phyla. However, the bacterial and archaeal analysis showed that the highest microbial diversity was found in the microalgal feedstock (Figure S1) due to the fact that it is not a pure *Scenedesmus* culture but it contains a minor proportion of bacteria and archaea (<10%). Even though there are a number of variables that differ between the reactor environments like SRT, HRT, and reactor configuration, the operational temperature is a key factor that has a large impact on the microbial community by determining which species can thrive [33]. The feedstock samples cluster distinctly from the reactor samples (Figure 2a) indicating that the communities present in both reactors were not influenced by feedstock microbes but are instead a result of the unique conditions present in each reactor (Figure 2b). An ANOSIM test indicated strongly significant dissimilarity between reactor types and the feedstock (Figure S2 and Table S1).

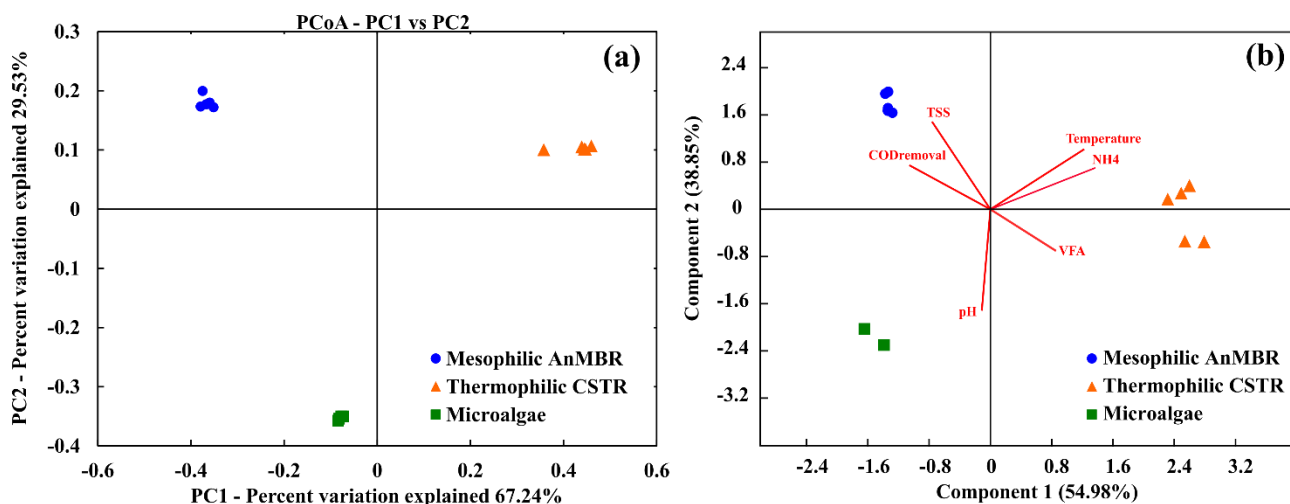


Figure 2. PCoA ordination of weighted UniFrac distances for the microbial community (a) and PCA analysis of process parameters listed as vectors (red) in the panel (b) for mesophilic AnMBR, thermophilic CSTR and microalgae.

3.2.1. Microbial community composition in the mesophilic AnMBR

The results of microbial community analysis revealed that Chloroflexi (27.9%), WWE1 (19.0%) and Proteobacteria (15.4%) were the most abundant phyla within the domain Bacteria, followed by Spirochaetes (7.7%), Bacteroidetes (5.9%) and Firmicutes (3.6%) (Figure 3). Generally, these phyla are abundant in anaerobic systems [34] although the substrate and the operational conditions allow particular phyla to dominate [14]. The candidate division T78 of the family Anaerolinaceae comprised most of the Chloroflexi phylum (Table 3, Figure S3). Chloroflexi can be highly abundant in some anaerobic digestion systems [34,35], playing an important functional role in anaerobic processes. Specifically, T78 is involved in the degradation of carbohydrates as a substrate for growth [35] and is able to use lignocellulosic biomass [36], both constituents of the microalgal cell wall. Moreover, bacteria in the phylum Chloroflexi can dominate in an anaerobic system when the ammonia concentration is low [2,37], which is consistent with the ammonia levels present in the mesophilic AnMBR (Figure 1c). The WWE1 phylum was mostly comprised of the candidate division W22 belonging to the family Cloacamonaceae (Table 3, Figure S3), which is more than 99% similar to *Candidatus Cloacamonas acidaminovorans* [38]. The high abundance of this

phylum was previously reported in several anaerobic systems [35,39]. W22 are fermentative bacteria that obtain their energy mainly from the fermentation of amino acids to produce CO₂ and H₂, and also from the fermentation of sugars to generate acetate. Therefore, this bacteria is likely involved in syntrophic metabolism with hydrogen consumers such as methanogens, sulfate-reducing bacteria or acetogenic bacteria [38], and could contribute to either acetoclastic or hydrogenotrophic methanogenesis. The high presence of *Candidatus Cloacamonas acidaminovorans* in the mesophilic AnMBR could be a consequence of the sugars and amino acids produced from hydrolysis of the cellulose and proteins that constituted the structure of the *Scenedesmus* [6]. Proteobacteria were mostly composed of the genera *Syntrophus* in the Deltaproteobacteria class (Table 3, Figure S3). This is one of the most abundant bacteria in the anaerobic digestion process due to its ability to syntrophically oxidize propionate to acetate, which is subsequently used by acetoclastic methanogens [40].

Bacteria of the Bacteroidetes phylum play an important role in cellulose and protein degradation by producing propionate and acetate as fermentation products [39]. However, the abundance of this phylum in the mesophilic AnMBR is lower than previously reported [41]. The phylum Spirochaetes was found to be a consumer of intermediate metabolites such as glucose [40]. Bacteria of the phylum Firmicutes have the ability to degrade organic compounds such as polysaccharides [42]. Nevertheless, in the AnMBR, the abundance of this phylum is lower than reported in previous studies [43], which may be related to the feedstock, the low ammonium concentration that benefits Chloroflexi growth and the use of the anaerobic membrane since membrane technology allows retention of slow-growing species such as Chloroflexi [44,37] and methanogenic microorganisms [45].

The AnMBR exhibited a high diversity of phyla with cellulolytic and proteolytic capabilities which

are responsible for degrading the microalgal biomass, and this could explain the high levels of organic matter removal achieved in this reactor (Figure 1a). The relative abundance of the phyla detected were quite different than what previous studies have reported. Whereas Bacteroidetes was found to be the dominant phylum within a mesophilic CSTR treating *Scenedesmus obliquus* [14], Sanz et al. [46] detected Proteobacteria as the major phylum when *Chlorella* microalgal biomass was degraded anaerobically within a mesophilic CSTR. However, neither of these phyla were dominant in the present study. Moreover, the AnMBR leads to higher microbial diversity than observed previously in microalgal digesters. The Simpson index (Table 4), which reflects both the number of species and the evenness of their abundance distribution in a sample, was lower than the values reported by Seo et al. [47] who digested *Ettlia* microalgal residue within a mesophilic CSTR, but also the Simpson, Shannon and Gini indices (Table 4) supported higher diversity in the AnMBR than values reported by Sanz et al. [46] for anaerobic digestion of *Chlorella* within a CSTR (0.09, 3.93 and 0.93, respectively). These results suggest that mesophilic reactors operated at high SRT enabled with membrane technology promote retention of microorganisms with a low growth rate, which creates a unique microbial community capable of degrading the *Scenedesmus* cell-wall, thereby resulting in one of the highest biogas production currently reported from the anaerobic digestion of raw *Scenedesmus* spp. grown on wastewater. The highest biogas production previously reported was obtained by Tartakovsky et al. [48], who achieved a biodegradability of 52-53% during the anaerobic digestion of a pure *Scenedesmus* sp. AMDD culture within a mesophilic CSTR at 16 and 58 days of HRT.

The Euryarchaeota phylum comprised 5.4 % of all sequences (Figure 3 Table 3) and mostly consisted of the *Methanosaeta* genus belonging to the Methanosarcinales order (83.6%), while Methanobacteriales and Methanomicrobiales constituted 7.4% and 5.6% of the phylum, respectively (Table 3). *Methanosaeta* are acetoclastic methanogens whose high abundance could

indicate that the most important pathway to methane production is via acetoclastic methanogenesis. This result is in accordance with the high presence of Chloroflexi, WWE1 and Proteobacteria phyla, and with the absence of VFAs in the effluent from the AnMBR (Table 2). However, although to a minor extent, hydrogenotrophic methanogenesis contributes to the methane production via Methanobacteriales and Methanomicrobiales, and the syntrophic metabolism of *Candidatus Cloacamonas acidaminovorans* suggests it may be involved in the process. These results regarding the Archaeal domain are in contrast with Yu et al. [45], who found that the hydrogenotrophic pathway was the dominant methanogenic process in a mesophilic AnMBR treating waste activated sludge. However, Ellis et al. [15] found acetoclastic methanogenesis as the main pathway to methane production in a microalgal anaerobic digester, whereas Wirth et al. [14] and Nolla-Ardèvol et al. [49] detected *Methanosarcina* as the most abundant Archaea in a mesophilic CSTR treating *Scenedesmus obliquus* and *Spirulina*, respectively. These results seem to indicate that the methanogenic pathway is determined by the sludge characteristics such as ammonium, free ammonia and temperature rather than the feedstock used or the reactor configuration.

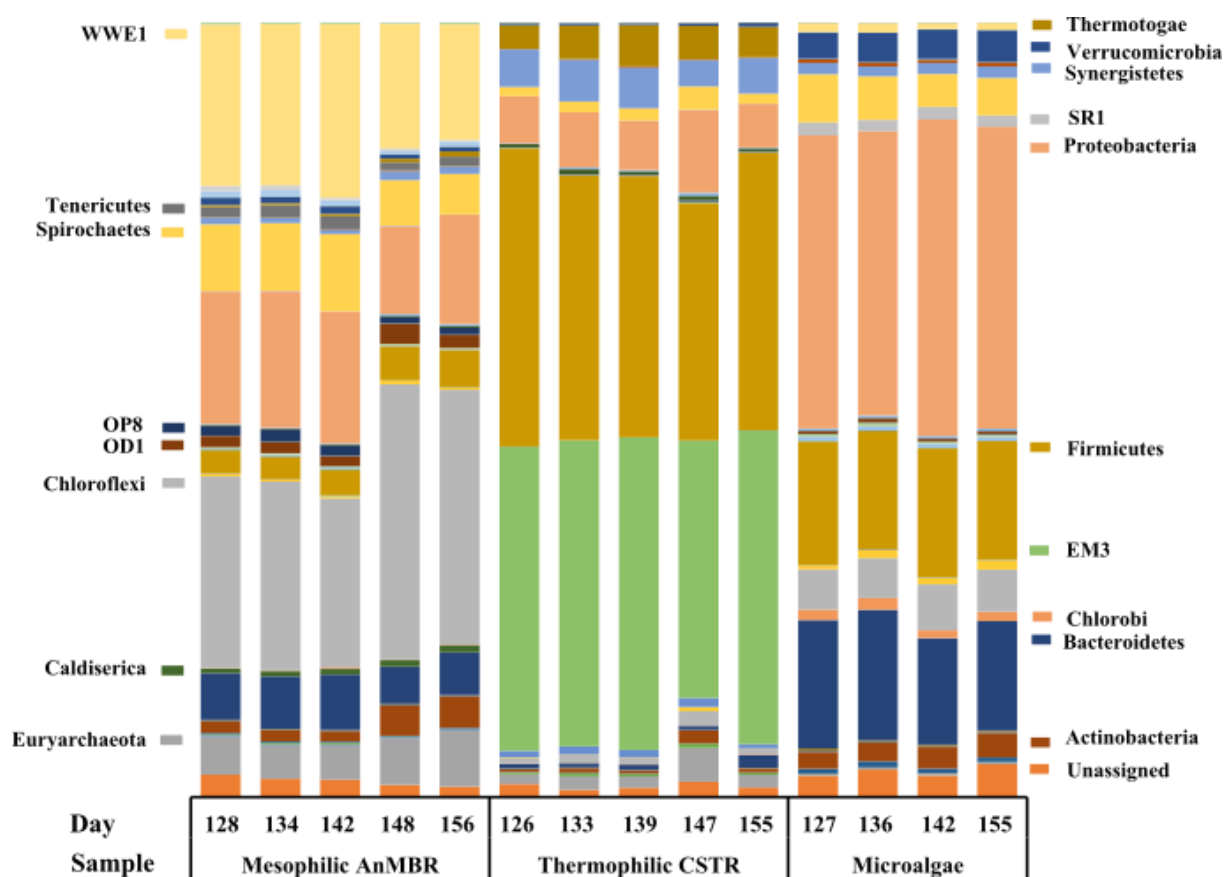


Figure 3. Relative abundance of Bacterial and Archaeal OTUs at the phylum level. Taxonomic groups with relative abundance lower than 1% were excluded from the plot legend flanking the bars.

Table 3. The most abundant Bacterial and Archaeal genera in the mesophilic AnMBR, Thermophilic CSTR and microalgal feedstock.

Mesophilic AnMBR					
Bacteria domain (92.50%)					
Phylum	Class	Order	Family	Genus	Relative abundance
WWE1	Cloacamonae	Cloacamonales	Cloacamonaceae	W22	18.67
Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	T78	16.27
Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae		4.33
Chloroflexi	Anaerolineae	GCA004			1.03
Chloroflexi	Anaerolineae	SJA-15			1.25
Spirochaetes	Spirochaetes	Spirochaetales	Spirochaetaceae	<i>Treponema</i>	7.47
Bacteroidetes	Bacteroidia	Bacteroidales			4.81
Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophaceae	<i>Syntrophus</i>	3.59
Proteobacteria	Gammaproteobacteria	Thiotrichale	Thiotrichaceae	<i>Thiothrix</i>	2.17

OD1					1.65
Tenericutes	Mollicutes	Acholeplasmatales	Acholeplasmataceae	<i>Acholeplasma</i>	1.31
Firmicutes	Clostridia	SHA-98			1.03

Archaea domain (5.39%)

Phylum	Class	Order	Family	Genus	Relative abundance
Euryarchaeota	Methanomicrobia	Methanosarcinales	Methanosaetaceae	<i>Methanosaeta</i>	4.49
Euryarchaeota	Methanomicrobia	Methanomicrobiales			0.30
Euryarchaeota	Methanobacteria	Methanobacteriales	WSA2		0.19

Thermophilic CSTR

Bacteria domain (96.49%)

Phylum	Class	Order	Family	Genus	Relative abundance
EM3					38.56
Firmicutes	Clostridia	Thermoanaerobacterales	Thermodesulfobiaceae	<i>Coprothermobacter</i>	30.10
Firmicutes	Clostridia	Clostridiales	Christensenellaceae		0.76
Firmicutes	Clostridia	Clostridiales	Syntrophomonadaceae	<i>Syntrophomonas</i>	0.62
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		0.46
Synergistetes	Synergistia	Synergistales	Anaerobaculaceae	<i>Anaerobaculum</i>	4.22
Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	PD-UASB-13	0.15
Thermotogae	Thermotogae	Thermotogales	Thermotogaceae	<i>Fervidobacterium</i>	3.68
Thermotogae	Thermotogae	Thermotogales	Thermotogaceae	S1	0.51
Spirochaetes	Spirochaetes	Spirochaetales	Spirochaetaceae	<i>Treponema</i>	1.62
Proteobacteria	Alphaproteobacteria	Rhizobiales			0.88
Proteobacteria	Gammaproteobacteria	Thiotrichales	Thiotrichaceae	<i>Thiothrix</i>	0.74

Archaea domain (2.09%)

Phylum	Class	Order	Family	Genus	Relative abundance
Euryarchaeota	Methanobacteria	Methanobacteriales	WSA2		1.39
Euryarchaeota	Methanomicrobia	Methanosarcinales	Methanosaetaceae	<i>Methanosaeta</i>	0.62
Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanospirillaceae	<i>Methanospirillum</i>	0.05

Microalgae

Bacteria domain (96.38%)

Phylum	Class	Order	Family	Genus	Relative abundance
Proteobacteria	Alphaproteobacteria	Rickettsiales			6.69
Proteobacteria	Gammaproteobacteria	Thiotrichales	Thiotrichaceae	<i>Thiothrix</i>	5.57

Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfomicrobiaceae	<i>Desulfomicrobium</i>	2.60
Proteobacteria	Alphaproteobacteria	Rhizobiales			2.49
Bacteroidetes	Bacteroidia	Bacteroidales			5.33
Bacteroidetes	Saprospirae	Saprospirales	Saprospiraceae		2.45
Spirochaetes	Spirochaetes	Spirochaetales	Spirochaetaceae	<i>Treponema</i>	4.49
Firmicutes	Clostridia	Clostridiales	Acidaminobacteraceae	<i>Fusibacter</i>	4.05
Firmicutes	Clostridia	Clostridiales	Christensenellaceae		3.00
Firmicutes	Clostridia	Clostridiales			1.85
Chloroflexi	Anaerolineae	Caldilineales	Caldilineaceae		1.86
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	<i>Luteolibacter</i>	1.85
Archaea domain (0.28%)					
Phylum	Class	Order	Family	Genus	Relative abundance
Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanoregulaceae		0.13
Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanospirillaceae	<i>Methanospirillum</i>	0.09

Table 4. Mean \pm Standard Error of diversity indices obtained from the mesophilic AnMBR and thermophilic CSTR both fed with *Scenedesmus* microalgal biomass.

	Mesophilic AnMBR	Thermophilic CSTR
Simpson	0.012 \pm 0.001	0.050 \pm 0.005
Shannon	6.518 \pm 0.168	4.039 \pm 0.396
Gini	0.971 \pm 0.002	0.987 \pm 0.003
Observed OTUs	1396 \pm 73	977 \pm 96

3.2.2. Microbial community in thermophilic CSTR

EM3 (38.7%) and Firmicutes (34.6%) were the dominant phyla in the thermophilic reactor (Figure 3), followed by Proteobacteria (7.2%), Synergistetes (4.7%) and Thermotogae (4.2%). Firmicutes is one of the most frequently observed phyla in thermophilic anaerobic digesters due to its ability to hydrolyse protein and polysaccharides [42]. The class Clostridia comprised 94.3% of the phylum

Firmicutes, which also consisted mostly of the genera *Coprothermobacter* (Table 3, Figure S3). The class Clostridia encompasses a range of bacteria able to degrade cellulose and to syntrophically oxidize acetate, and Clostridia occur in reactors with relatively high ammonium concentrations [50] such as the levels achieved in the thermophilic CSTR (Figure 1c). Clostridia commonly co-occur with hydrogenotrophic methanogens, which maintain a low H₂ concentration [51]. Specifically, the genera *Coprothermobacter* is a thermophilic bacteria with high proteolytic activity able to degrade proteins into acetate, H₂ and CO₂ [52], and thus its presence is likely related to its ability to degrade components of the microalgal cell-wall. *Scenedesmus* have a high proportion of proteins and cellulose in their cells [8], and this would explain the high proportion of proteolytic and cellulolytic bacteria observed in the reactors. In contrast to the mesophilic AnMBR, the phylum Chloroflexi only accounted for 1.1% of all reads while the phylum Firmicutes is one of the dominant phyla in the thermophilic CSTR. The distribution of these phyla may be attributed to the operational temperature and to the low tolerance of Chloroflexi to ammonium, which was almost 2-fold higher in the thermophilic CSTR than in the mesophilic AnMBR (Table 2). Nevertheless, the functional role of the Chloroflexi could be fulfilled by the phyla Firmicutes and EM3 as both seem to exhibit functional overlap [35].

The uncharacterized phylum EM3 was recently assigned (based on 98% 16S rRNA identity) a new, metagenome-derived candidate member named “Pyropristinus” Type 1 and Type 2 [53]. The EM3 are suggested to be chemoorganoheterotrophic bacteria whose putative role in hot springs is to degrade polysaccharides and proteins (such as those found in the microalgal cell-wall used as a feedstock in the present contribution) and to utilize endogenous and exogenous organic carbon sources in their metabolism. Colman et al. [53] concluded that EM3 are likely to perform aerobic respiration and oxidative phosphorylation in the hyperthermophilic temperature range (70°C-90°C). However, the authors found that EM3 plays an important role in carbon cycling and suggested that

this phylum could potentially respire anaerobically, which is confirmed due to the high abundance of this phylum in the thermophilic CSTR in the present study, and its detection also in the thermophilic digestion of lignocellulosic biomass [54].

Proteobacteria, which are also present in the mesophilic AnMBR, are involved in cellulose and protein degradation as well as the syntrophic degradation of organic acids. Likewise, the *Anaerobaculum* genus within the Synergistetes phylum is also involved in the syntrophic degradation of organic acids, peptides and carbohydrates, and the members of the Thermotogae phylum are able to syntrophically oxidize acetate [55]. Consequently, their low abundance may be associated with high VFA concentration in the reactor (Table 2). The high proteolytic activity of the thermophilic CSTR may determine the microalgal degradation capacity of the process.

Despite the fact that the diversity estimated in the thermophilic CSTR was lower than in the mesophilic AnMBR (Figure S1 and Table 4), the thermophilic CSTR also contained phyla with high hydrolytic potential. Whereas the genus *Coprothermobacter* is commonly detected in thermophilic reactors and especially when cellulose is used as feedstock, EM3 is a candidate phylum that was observed in high abundance but whose functional role in anaerobic digesters remains undefined. However, the high abundance of EM3 (38.7%) in the present study suggests that this phylum plays an important role in the biodegradation of microalgae. Further studies are needed to define the functional metabolism of EM3, which could lead to insights as to further optimisation of the process.

The Archaeal domain accounted for 2.1% of all reads among thermophilic CSTR samples (Figure 3, Table 3) and shows that the most abundant Archaea belong to the order Methanobacteriales (67% of Archaea) whose versatile metabolism allows them to perform either hydrogenotrophic or

acetoclastic methanogenesis. However, the high abundance of hydrogen producers and syntrophic acetate oxidizing microorganisms among the Bacterial phyla present in the thermophilic CSTR suggest that methane was mainly produced by the hydrogenotrophic pathway. Nevertheless, strict acetoclastic methanogens such as *Methanosaeta* (30.1%) are also present. The high ammonium ($463.7 \text{ mgNH}_4\text{-N}\cdot\text{L}^{-1}$) and free ammonia ($30.68 \text{ mgNH}_3\text{-N}\cdot\text{L}^{-1}$) concentrations in the thermophilic CSTR likely influence the methanogens present as such conditions are known to promote the growth of Methanobacteriales [49], and its presence suggests that methane was produced through the hydrogenotrophic pathway.

3.2.3. Bacterial and Archaeal community in *Scenedesmus* microalgal biomass

The analysis of the Bacterial and Archaeal communities in the feedstock was performed in order to evaluate their influence on the microbial community of the reactors given that the microalgal feedstock was not a pure culture. This analysis revealed that only 0.27% of all reads were assigned to the Archaeal domain (Table 3) while the Bacterial domain is comprised of the phyla Proteobacteria (38.6%), Firmicutes (15.8%), Bacteroidetes (15.3%), Chloroflexi (5.4%), Spirochaetes (4.0%), Verrucomicrobia (3.7%) and Actinobacteria (2.6%) (Figure 3). The substrate and the reactors shared some Bacterial phyla (Figure 3) although their relative abundances were very different as well as the particular genera found within the shared phyla (Table 3 and Figure S3). Beta-diversity (Figure 2a) and ANOSIM analysis (Table S1 and Figure S2) confirmed that the microbial communities in both reactors are very distinct from the one found within the microalgal feedstock. These results indicate that the microbial communities unique to each reactor were a consequence of reactor configuration and operational conditions established. In relation to other studies, the use of recalcitrant *Scenedesmus* spp. biomass as the reactor feedstock is likely to have selected for species adapted to the biodegradation of this substrate.

4. CONCLUSIONS

The anaerobic digestion of *Scenedesmus* microalgal biomass reached 70% COD removal under mesophilic conditions using an AnMBR. The microbial community analysis performed during steady state operation of this reactor revealed a wide variety of phyla including Chloroflexi, WWE1 and Proteobacteria. Since these phyla are previously known to exhibit high hydrolytic capacity, they were likely implicated in breakdown of the microalgal cell wall. The diverse phyla that contributed to microalgal degradation are probably a consequence of the high SRT established in the reactor by using an AnMBR reactor configuration, whose membrane technology is able to retain slow-growing microbes within the system. Regarding the domain Archaea, *Methanosaeta* was the dominant archaeal genera which suggests that the organic matter was mainly transformed to methane by the acetoclastic pathway. *Methanosaeta* predominance would be driven by the free ammonia concentrations as well as the operational temperature.

The anaerobic digestion of the same microalgae within the thermophilic CSTR reached 32.6% COD removal and ammonia concentration was almost 2-fold higher than in the mesophilic AnMBR. The bacterial analysis revealed that the diversity was lower in the thermophilic CSTR than in the mesophilic AnMBR, which is likely to be related to the operational temperature. Nonetheless, in the thermophilic CSTR bacteria with high proteolytic and cellulolytic capabilities were detected such as Firmicutes and EM3. The highly abundant EM3 (38.7%) indicates that this phylum could be involved in the microalgal degradation, although its functional role in anaerobic digesters is still undefined. As this phylum appears to be important for biogas production during the thermophilic anaerobic digestion of microalgae, further studies are needed in order to characterize its metabolism and to optimize the biological process. The abundant syntrophic bacteria together with hydrogenotrophic methanogens and high reactor ammonia concentrations suggest that the hydrogenotrophic pathway should be the principal mechanism for methane production under these

conditions.

In conclusion, this study demonstrates the efficient conversion of *Scenedesmus* microalgal biomass to biogas through the use of an AnMBR configuration that permits extended residence time of the biomass and its associated hydrolysing microbes within the reactor. The unique substrate and anaerobic digester configurations resulted in correspondingly unique microbial communities, including a thermophilic CSTR community dominated by the uncultured EM3 candidate phylum. Hence, we have identified microbes involved in the degradation of *Scenedesmus* biomass, and it may now further explore their metabolic activity through shotgun metagenomic and metaproteomics. Optimisation of the biodegradation process could be achieved by tailoring operational conditions to enhance growth of the favoured microbes. Likewise, membrane technology is a suitable approach able to operate the reactor at high SRT with a reduced surface requirement and thereby maintains high microbial diversity that promotes the hydrolysis of normally recalcitrant microalgae.

CONFLICT OF INTEREST

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

CONTRIBUTIONS

SG: Performed experiments, analysed data, drafted and wrote the paper. JCG: Planed and designed the experiments, analysed microbial data, wrote the article. DA: Planed and designed the experiments and analysed anaerobic process data. JF: Planed and designed the experiments, analysed anaerobic process data. AS: Planed and designed the experiments, analysed anaerobic

process data. SJH: Planed and designed the experiments, analysed data, wrote the article.

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SUPPLEMENTARY DATA

The following is the supplementary data related to this article:

Figure S1. Richness of the microbial community in both reactors and the algal feedstock. The figure legend indicates the colour for each experimental group. Richness is expressed as the number of observed OTU_{0.97} as determined from 16S amplicon analysis. Bars indicate the Standard Error (SE).

Figure S2. Between-group vs. within-group distances according to the ANOSIM statistical test.

Figure S3. Relative abundance of bacterial and archaeal OTUs at the genus level. Taxonomic groups with relative abundance lower than 1% were excluded from the plot legend flanking the bars.

Table S1. R value matrix from the ANOSIM test. R = 1 indicates that the within-group similarity is greater than the between-group similarity.

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