Effect of long residence time and high temperature over anaerobic biodegradation of *Scenedesmus* microalgae grown in wastewater

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Abstract

Anaerobic digestion of indigenous *Scenedesmus* spp. microalgae was studied in continuous lab-scale anaerobic reactors at different temperatures (35ºC and 55ºC), and sludge retention time – SRT (50 and 70 days). Mesophilic digestion was performed in a continuous stirred-tank reactor (CSTR) and in an anaerobic membrane bioreactor (AnMBR). Mesophilic CSTR operated at 50 days SRT only achieved 11.9% of anaerobic biodegradability whereas in the AnMBR at 70 days SRT and 50 days HRT reached 39.5%, which is even higher than the biodegradability achieved in the thermophilic CSTR at 50 days SRT (30.4%). Microbial analysis revealed a high abundance of cellulose-degraders in both reactors, AnMBR (mainly composed of 9.4% *Bacteroidetes*, 10.1% *Chloroflexi*, 8.0% *Firmicutes* and 13.2% *Thermotogae*) and thermophilic CSTR (dominated by 23.8% *Chloroflexi* and 12.9% *Firmicutes*). However, higher microbial diversity was found in the AnMBR compared to the thermophilic CSTR which is related to the SRT. since high SRT promoted low growth-rate microorganisms, increasing the hydrolytic potential of the system. These results present the membrane technology as a promising approach to revalue microalgal biomass, suggesting that microalgae biodegradability and consequently the methane production could be improved operating at higher SRT.

Keywords

Anaerobic digestion; Biodegradability; Membrane technology; Microalgae; Microbial analysis; *Scenedesmus* spp.

1. Introduction

In recent years, the search for renewable energy sources to replace fossil fuels has triggered intense research on microalgae, which are a potential feedstock for biofuel production (Ward et al., 2015). However, with the current technology, microalgal biomass cultivation for biodiesel production, which includes energy demanding operations such as biomass drying and lipid extraction by solvents, presents a negative energy balance (Lardon et al., 2009; Vassilev and Vassileva, 2016). This energy balance can be improved incorporating an anaerobic digestion step of the algal residue after oil

extraction, taking advantage of the cell wall disruption made during the extraction which facilitates the biological digestion (Caporgno et al., 2016; Sialve et al., 2009). Nevertheless, these authors highlight that anaerobic digestion of the whole biomass is more energetically favourable than its use for biodiesel production when the cell lipid content does not exceed 40%.

Likewise, in order to reduce the costs associated to the production of microalgal biomass, wastewater can be used as a source of nutrients for its cultivation, since microalgae can be grown on the effluent of anaerobic digestion processes (Mo and Zhang, 2013; Wang and Park, 2015). Combining microalgae cultivation with wastewater treatment allows obtaining effluents free of nutrients, avoiding the use of external nutrients for microalgae growth, and generating microalgal biomass to subsequently produce biogas. Wastewater composition and ambient conditions definitely influence the microalgae species that will predominate in ponds and photobioreactors such as *Scenedesmus* and *Chlorella* (Viruela et al., 2016). Therefore, to fully exploit the advantages of using wastewater for microalgae growth, the selection of the microalgae species according to its biogas production potential is not an option. A drawback appears when refractory microalgae species furnished with a rigid cell-wall such as *Scenedesmus* predominate (Mussgnug et al., 2010), since they make it difficult the hydrolysis step during anaerobic digestion and significantly limit the methane production.

Some authors have focused on improving biogas production by applying different pretreatment methods (such as a thermal, microwave and ultrasonic) to carry out microalgae cell-wall disruption (Alzate et al., 2012; Caporgno et al., 2016; González-Fernández et al., 2012). Nevertheless, the enhancement of methane production after pretreatments cannot offset the cost of energy or the chemical reagents required (Passos et al., 2014). An alternative way to improve the microalgae biodegradability is to increase the hydrolytic activity of anaerobic biomass, operating anaerobic biological processes at high solid retention time (SRT) in order to promote slow-growing microorganisms. For this purpose, although several reactor configurations have been already implemented to digest microalgae at moderate SRT, such as continuous stirred-tank reactor (CSTR) (Ras et al., 2011) or an upflow anaerobic sludge blanket (UASB) (Tartakovsky et al., 2015), a possible configuration to promote the hydrolytic activity involves the use of membrane technology. This technology allows complete retention of biomass within the system, decoupling the SRT and the hydraulic retention time (HRT), and therefore operate the digester at higher SRT than conventional anaerobic digesters. Nevertheless, up to now, very few studies have applied this configuration to the treatment of microalgae (Zamalloa et al., 2012b) and none of them have applied it on *Scenedesmus* spp. fresh microalgae. On the other hand, operational temperature is another alternative to significantly increase the hydrolytic activity of the system. Song et al. (2004) demonstrated that the thermophilic microbial population is related to a high enzymatic activity. However, thermophilic processes tend to be more instable due to an inhibition by free ammonia (Montingelli et al., 2015). Indeed, an important aspect to be considered during the anaerobic digestion is the microbial community involved in the process due to its key role in the optimization of the treatment. Nowadays, few studies have reported information about the microbial population that makes possible the degradation of microalgae feedstock: only mesophilic anaerobic digesters within CSTR configuration treating pure cultures of *Nannochloropsis salina* (Ma et al., 2015), *Spirulina* sp. (Nolla-Ardèvol et al., 2015), *Chlorella vulgaris* (Sanz et al., 2016) and

Scenedesmus obliquus (Wirth et al., 2015a) or a mixture culture of *Scenedesmus* sp. and *Chlamydomonas* sp. (Wirth et al., 2015b) have been massively sequenced. However, further studies are needed to elucidate the optimum microbial consortium that allows maximizing the valorisation of microalgal biomass as biogas.

On the basis of these approaches, the aim of this research is to present anaerobic membrane technology as a promising approach to revalorize a recalcitrant fresh microalgae such as *Scenedesmus* spp. through their biomethanization. For this purpose, in the present contribution, indigenous fresh *Scenedesmus* microalgae grown in wastewater were anaerobically treated within a conventional CSTR configuration under mesophilic and thermophilic conditions; as well as within a mesophilic anaerobic membrane bioreactor (AnMBR) configuration at a long residence time. Additionally, for a further comprehension of the anaerobic processes, a microbial community analysis with Next Generation Sequencing (NGS) was performed in order to establish one of the first characterization approaches of the microbial population involved in microalgal degradation, retrieving a valuable information from Illumina sequencing.

2. Materials and Methods
2.1. Reactors description and operational conditions
Algal anaerobic digestion was carried out in two water-jacketed laboratory-scale reactors (see Figure S1), a mesophilic CSTR-AnMBR and a thermophilic CSTR. The reactors were sealed to prevent any ingress of air and were equipped with probes for on-line measurement of pH, temperature, oxidation-reduction potential, gas pressure and gas flow rate. A data acquisition software was developed to continuously save and plot all the measured data. The mesophilic and thermophilic reactors were run for more than 300 and 160 days, respectively, and fed once a day every weekday. Both digesters were started up filling up the working volume with the appropriate inoculum, mesophilic or thermophilic sludge, and using fresh microalgae biomass as substrate from the beginning of the experiment. The start-up period corresponded to the first 3 months in both reactors and their operational conditions are summarized in Table 1.

Table 1. Operational conditions studied in mesophilic and thermophilic reactors.

	Mesophilic reactor		Thermophilic reactor
Experimental Period	CSTR	AnMBR	Thermo-CSTR
Temperature (ºC)	35	35	55
SRT (d)	50	70	50
HRT (d)	50	50	50
OLR (g $L^{-1}d^{-1}$)	0.2	0.2	0.2
Membrane	No	Yes	No

2.1.1. Mesophilic reactor
A CSTR of 11.5 L with 2.5 L of headspace was stirred by biogas recirculation and inoculated with sludge from a conventional mesophilic digester (35ºC) located at Carraixet WWTP (Valencia, Spain). The biomass was not recirculated, being the SRT equal to the hydraulic retention time (HRT), which was established at 50 days. The reactor was operated at 35ºC since anaerobic biomass used as inoculum is already acclimated to this temperature and thus, given the strong influence of this parameter on

the microbial metabolism, thermal effect on biological process performance is avoided (Kiran et al., 2016; Hagos et al., 2017).

After 150 days of operation, the reactor configuration was modified, connecting a 0.42 m^2 hollow-fibre ultrafiltration membrane module (PUR-ON® Koch Membrane Systems) to the reactor. This connection allowed decoupling SRT from HRT, and thus operating at 70 days of SRT while keeping the HRT at 50 days. The volume of the membrane module was 0.9 L with a fibre nominal pore size of 0.05 µm, and a fraction of biogas was recycled to the membrane module in order to reduce the biofilm formation. Moreover, sludge was continuously recirculated from the reactor to the membrane module to maintain the sludge homogenised. The membrane was programmed to operate under three different stages: Filtration, backwashed and relaxation. Once the reactor was purged, the filtration stage was running until the permeate volume set was achieved, which was necessary to control the HRT. Backwashed was carried out every 120 seconds of filtration, during 20 seconds. Relaxation stage was established provided that filtration and backwashed stages were not running. Membrane performance was monitored by measuring transmembrane pressure (TMP) values and fluxes along the experimental period. The low TMP values reached along the experimental period made a chemical cleaning unnecessary (see Figure S2). In the AnMBR configuration, the final total volume was 12.4 L with 2.5 L of headspace.

2.1.2. Thermophilic reactor
A CSTR of 2 L with 0.4 L of headspace was used for microalgae thermophilic digestion. The reactor was mixed with mechanical stirring and inoculated with sludge from a pilot thermophilic digester (Valladolid, Spain). The SRT and HRT was established at 50 days since biomass was not recirculated. The reactor was operated at 55ºC in order to minimize thermal effects on the biological process since inoculated microorganisms are already adapted to this temperature, as well as for representativeness due to this temperature is the most common value used in thermophilic digesters (Kiran et al., 2016; Mara and Horan, 2003).

2.2. Microalgae feedstock
Microalgae fed to the digesters was grown under stressed conditions in a membrane PhotoBioReactor (MPBR) pilot plant, located at Carraixet WWTP (Valencia, Spain). This microalgal biomass, mainly composed by *Scenedesmus* spp. microalgae (> 90%), was cultured for nutrient removal of the effluent from an AnMBR pilot plant that is treating real wastewater, as it was described by Viruela et al. (2016). Fresh microalgae were collected from MPBR pilot plant and concentrated by filtration to 10 g·L^{-1} to feed the lab-reactors with an organic loading rate (OLR) of 0.2 gCOD·L$^{-1}_{reactor}$·day^{-1} (Table 1). The mesophilic and thermophilic reactors were fed with the same influent.

2.3. Analytical methods
2.3.1. Process analysis
Ammonium, total nitrogen (N_{Tot}), sulphate, phosphate and total phosphorus (P_{Tot}) concentrations as well as total and soluble chemical oxygen demand (COD) and total and volatile suspended solids were measured according to Standard Methods (APHA, 2012). Volatile fatty acids (VFA) and alkalinity (ALK) were measured by titration using the method proposed by Moosbrugger et al. (1993). The methane fraction in biogas was periodically measured using a gas chromatograph fitted with a Flame Ionization Detector (GC-FID, Thermo Scientific).

The process efficiency was evaluated in terms of COD removal percentage (1) and biodegradability percentage (2).

$$\%COD_{removal} = [COD_{influent} - COD_{effluent}]/COD_{influent} \cdot 100 \qquad (1)$$

$$\%Biodegradability = CH_4\ produced\ /CH_4\ potential \cdot 100 \qquad (2)$$

where, $COD_{influent}$ (g·d^{-1}) is the COD of algae feed as substrate, $COD_{effluent}$ (g·d^{-1}) is the COD in the effluent of the reactors (purged sludge in the mesophilic and thermophilic CSTR, and permeated plus purged sludge in the AnMBR configuration), $CH_4\ produced$ (L·d^{-1}) is the methane generated in the anaerobic digestion of algae, directly related to organic matter degradation, and $CH_4\ potential$ (L·d^{-1}) is calculated theoretically considering the current reactor temperature and pressure, and that 350 mL of methane are produced per gram of COD (at 0ºC and 1 atm).

2.3.2. Microbial population analysis

Sludge homogenized samples were collected after 287 and 170 days of operation from mesophilic AnMBR and thermophilic CSTR, respectively, and stored immediately at -20ºC. Genomic DNA was extracted from each sample using E.Z.N.A Soil DNA Kit (Omega-Biotek) according to manufacturer's protocol. The concentration and purity of the genomic DNA extracted was determined by measuring the absorbance at 260 and 280 nm wavelength in Nanodrop 2000 spectrophotometer (Thermo Scientific).

Libraries of V4 hypervariable region of 16S rRNA gene were generated from extracted genomic DNA, following the procedure from Caporaso et al. (2011). For this purpose, 0.2ng/µl of purified DNA were used, after fluorometric quantification in a Qubit 3.0 fluorometer (Life Technologies). The multiplexing step was performed within Nextera XT Index Kit (Illumina). The libraries were sequenced in a 2x300 bp paired-end run within MiSeq Reagent kit v3 on a MiSeq Sequencer, according to manufacturer's instructions (Illumina) by *Fundación para el Fomento de la Investigación Sanitaria y Biomédica de la Comunidad Valenciana* (FISABIO). Sequence data were trimmed and filtered applying prinseq-pl algorithms (Schmieder and Edwards, 2011), post processed by merging the paired-end reads according to fastq-join default parameters (Aronesty, 2011) and then checked for chimeras (Edgar et al., 2011). Finally, a total amount of 18925 and 55195 reads from mesophilic and thermophilic samples (293±11 and 292±7 bp mean length), respectively, were classified up to *genus* level with Ribosomal Database Project's Classifier tool (Cole et al., 2009) into 568 and 904 *genera*-based taxonomic units with a default confidence-threshold of 0.8. Raw sequences data were deposited on the NCBI database with BioProject accession PRJNA339420.

2.3.3. Statistical analysis

The reactors were compared by ANOVA test using SPSS software (IBM SPSS Statistics 22.0) once the pseudo-steady state was achieved. ANOVA test was performed using 5 data values from every reactor (N=5) and considering the level of statistical significance (p-value) as 0.05.

All the analyses were carried out in triplicate in order to calculate the average and the standard deviation showed in tables and graphs.

In terms of microbial analysis, rarefaction curves were generated with vegan package v.2.3-1 in R software (version 0.99.489) (Oksanen et al., 2017), by plotting the number of sequences retrieved against the number of families identified (Figure S3) in order to show that the plateau phase was reached in both digester samples and therefore, the sequencing depth obtained through Illumina sequencing allowed the analysis of microbial population in the present study.

3. Results and discussion

3.1. Microalgae feedstock composition

Based on the weekly characterization of the microalgal biomass, average composition of the feedstock is shown in Table 2. The particulate mass ratios N/COD and P/COD means were 0.05 and 0.009 respectively, which are similar to the nutrient ratios 0.04268 and 0.00858 that can be obtained from the average composition of microalgae given by $CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$ (Grobbelaar, 2007). This reflects the nutrient recovery potential if anaerobic digestion of microalgal biomass achieves high biodegradation levels, since it would release a high amount of nutrients (Wang et al., 2013). However, it should be noted the presence of high total nitrogen content (Table 2) which could inhibit anaerobic digestion process as a result of high free ammonia concentration (Kwietniewska and Tys, 2014).

Table 2. Mean and standard deviation (SD) of the influent composition in mesophilic CSTR and AnMBR as well as in thermophilic CSTR.

Component (units)	Mean	(SD)	Component (units)	Mean	(SD)
COD (mg O_2 L^{-1})	10000	($\pm$435)	P_{Tot} (mg P L^{-1})	100	($\pm$8)
TSS (mg TSS L^{-1})	7151	($\pm$596)	NH_4^+ (mg NH_4-N L^{-1})	27.3	($\pm$4.2)
VSS (mg VSS L^{-1})	6481	($\pm$429)	PO_4^{-3} (mg PO_4-P L^{-1})	10.1	($\pm$2.2)
N_{Tot} (mg N L^{-1})	590	($\pm$35)	SO_4^{-2} (mg SO_4-S L^{-1})	100.0	($\pm$8.2)

3.2. Biological process performance

3.2.1. Effect of SRT

Effect of long residence time was evaluated by comparing a conventional mesophilic CSTR with a mesophilic AnMBR under the operational conditions shown in Table 1. The time course evolution of both mesophilic reactor configurations was monitored in terms of: methane percentage in the biogas and biogas flow rate, COD in the influent, COD evolution in the reactor and COD removal percentage, ammonia in the influent and in the reactor as well as the free ammonia present in the system, volatile fatty acids and alkalinity in the reactor, and reactor suspended solid concentration (Figure 1). As it can be seen in Figure 1a, the lowest methane production occurred in mesophilic CSTR, resulting in 11.9% of biodegradability (Table 3) and in a biogas production of 2.26 mL·$gVSS_{reactor}^{-1}$ with a relatively constant percentage of methane of 65.3%. During a start-up period of 3 months, a continuously accumulation of COD (Figure 1b) and TSS (Figure 1c) in the reactor was detected as probably consequence of the microorganisms adaptation to the experimental conditions established (Table 1) and to the substrate.. This low biodegradability reached in mesophilic CSTR is similar to the 9.4% reported by González-Fernández et al. (2013) for untreated *Scenedesmus* spp. biomass, which demonstrates again the high resistance of these microalgae to anaerobic digestion. To increase microalgae biodegradability, mesophilic reactor configuration was modified by the incorporation of a hollow-fibre ultrafiltration membrane module to the reactor that

allows decoupling SRT from HRT and therefore, operating at higher SRT keeping the treatment flow rate (Table 1). The TMP values remained lower than 0.08 bar (see Figure S2) with an average flux of 12.5 $L \cdot m^{-2} \cdot h^{-1}$ during each filtration period, operating with a maximum mixed liquor suspended solids (MLSS) of 6480 $mg \cdot L^{-1}$.

Once the process trended towards to stability, after incorporating the membrane in the experimental set-up (around day 270), COD removal significantly increased from 11.4 to 40.4% (Figure 1b), although the OLR was maintained at 0.2 $g \cdot d^{-1} \cdot L^{-1}$. The new experimental conditions set up after membrane addition (Table 1) caused a higher concentration of VFA available for methanogenic *Archaea*, which contributed to an initial increase of biogas production (Figure 1a). However, hydrolytic bacteria have a higher growth rate than methanogenic *Archaea* and therefore, their quickly adaptation to the new operational conditions contributed to a slight accumulation of VFA (Figure 1e). As the process progressed, the VFA decreased as a consequence of methanogenic *Archaea* adaptation and its subsequent methane production. Accordingly, biogas flow rate periodically increased until achieving the pseudo-steady state at day 267 (Figure 1a), which is mainly associated to the effect of increasing the SRT. The higher retention of the anaerobic biomass in the system promoted low growth-rate microorganisms, involved in microalgae degradation. Consequently, as these microorganisms grew, a higher substrate hydrolysis occurred and therefore, biogas production increased. Once pseudo-steady state was reached, biogas production increased 4-fold, until 8.91 $mL \cdot gVSS_{reactor}^{-1}$ (Figure 1a), resulting in methane yield increased from 35.7 to 148.5 $mLCH_4 \cdot gCOD_{inf}^{-1}$ (Table 3) and significantly improving the biodegradability from 11.9 to 39.5%. This hydrolytic increase can be also observed in the TSS trend, which did not significantly increase in the reactor after increasing the SRT (Figure 1c, Table 3), confirming the improvement in the microalgal biodegradability due to hydrolytic activity was promoted. Previous studies reported a degradation efficiency of 52-53%, digesting a pure *Scenedesmus* sp. AMDD culture in a anaerobic conventional CSTR reactor, 1.3-fold higher than our results (Tartakovsky et al., 2013). However, these authors did not detect any improvement when increased the SRT from 16 to 58 days. Likewise, *Scenedesmus obliquus* was treated by hybrid flow-through system under mesophilic conditions, achieving conversions efficiencies of 26% (Zamalloa et al., 2012a). Ras et al. (2011) found that biogas production through anaerobic digestion of *Chlorella vulgaris* was increased 4-fold, when SRT was increased from 16 to 28 days, achieving a 51% of COD removal. As these studies show, microalgae biodegradability varies considerably which might be associated to cell wall composition of each specie and the different cultivations conditions of the microalgal biomass (Dębowski et al., 2013; Frigon et al., 2013). Consequently, the optimization of the anaerobic process, in order to maximize the energy recovery, strongly depends on the specific microalgae strain.

As was expected, anaerobic digestion of microalgae released nutrients (N and P) to the soluble phase, which could be either recovered or recycled to the culture medium for additional microalgae growth. As can be seen in Figure 1d, despite ammonium concentration in microalgae feed is low, reactor presented ammonium as the main compound of the soluble nitrogen in the effluent. In both mesophilic configurations, CSTR and AnMBR, nitrogen mineralization exceeded the anaerobic biodegradation percentages, achieving 34% and 55% in CSTR and AnMBR, respectively. These results seem to reflect partial degradation of microalgae cells, being low the nitrogen content of the slowly digestible organic residues that remain after digestion. The higher rate of

nitrogen solubilisation observed in the AnMBR compared with CSTR confirms a greater degradation due to the higher SRT. The high protein content of *Scenedesmus* biomass (Biller and Ross, 2014) can lead to anaerobic digestion inhibition as a result of high free ammonia concentration. However, Figure 1d shows that in both configurations free ammonia concentrations remained lower than 20 mg·L^{-1} and VFA concentration lower than 10 mg·L^{-1} with high ALK values (Figure 1e), confirming that this inhibition did not occur. Different studies have found that methanogenic activity is not affected at these low NH_3 concentrations (Garcia and Angenent, 2009; Siles et al., 2010; Sung and Liu, 2003). A high VFA concentration was only detected in the start-up of the mesophilic CSTR, which was a consequence of the acclimation of the inoculum to the new experimental conditions set up (Figure 1e).

Nevertheless, despite the fact of using an AnMBR increases the biodegradability 3.4-folds, further studies are needed in order to enhance the biogas production and consequently, the energy efficiency of the process (Zamalloa et al., 2011). The results obtained in this study suggested that an AnMBR operated at SRT higher than 70 days could improve the microalgae biodegradation since higher hydrolytic percentage could be reached because of the microorganisms promoted in the system, as it will be discussed later. Some alternatives to operating the reactors at high SRT could be the bioaugmentation of the anaerobic microbial community with specific cellulolytic microorganisms in order to increase the hydrolytic potential of the anaerobic biomass; a co-digestion of the microalgae with primary sludge or food waste; or a pretreatment of the microalgal biomass by applying thermal or enzymatic pretreatments provided their costs are minimised.
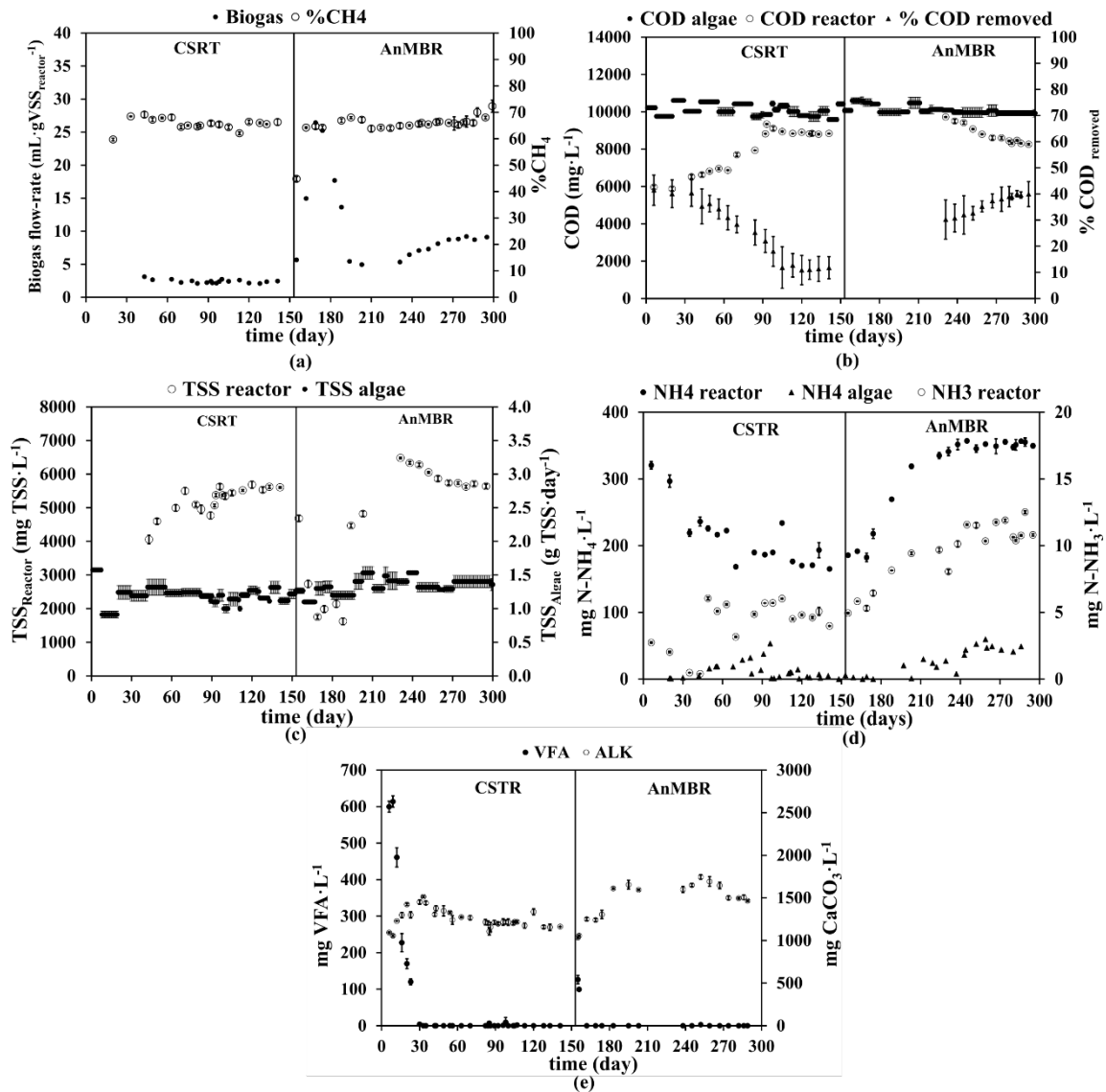
Figure 1. Temporal evolution along the experimental study in mesophilic conditions of: **(a)** biogas production per gram of VSS in the reactor and methane percentage **(b)** COD concentration in the influent, in the reactor and COD removed percentage **(c)** suspended solids in the influent and in the reactor **(d)** ammonia concentration in the influent and in the reactor and **(e)** volatile fatty acids and alkalinity in the reactor. The vertical solid line indicates the reactor configuration change.

Table 3 summarizes the results obtained in each experimental period at pseudo-steady state. In this table, it can be seen that the highest microalgae biodegradability under mesophilic conditions was achieved within AnMBR configuration. This fact confirms that the increment in the SRT by the total retention of biomass in the system using membrane technology, increases the hydrolytic activity of the sludge with a consequently enhancement in the biogas production.

Table 3. Average biodegradability, methane production and main digestate characteristics obtained at pseudo-steady state during semi-continuous digestion of fresh *Scenedesmus* spp. biomass.

	Mesophilic reactor		Thermophilic reactor
	CSTR	AnMBR	Thermo-CSTR
Methane yield (mL$_{CH4}$ gCOD$_{inf}$ $^{-1}$)	35.7 ±5.1	148.5 ±4.6	105.3 ±2.8
Biogas flow rate (mL gVSS$_{reactor}$$^{-1}$)	2.3 ±0.2	8.9 ±0.2	7.1 ±0.5
CH$_4$ % in biogas	67.0 ±5.8	69.4 ±4.8	70.0 ±3.1
% biodegradation [% biomethanization][1]	11.9 ±3.4 [9.9 ±3.2]	39.5 ±0.5 [37.8 ±2.2]	30.4 ±1.7 [29.4 ±1.2]
Digestate characteristics:			
TSS (mg TSS L^{-1})	5593 ±61	5690 ±48	5667 ±97
VSS (mg VSS L^{-1})	4843 ±92	4791 ±74	3512 ±109
sCOD [TVFA] (mg L^{-1})	18.5 ±4.3 [<10]	26.4 ±6.8 [<10]	611 ±18 [145 ±4]
NH$_4^+$ (mg L^{-1}) [% N solubilisation]	188 ±15 [33.6 ±2.8]	350 ±30 [55.6 ±2.3]	382 ±37 [63.7 ±1.3]
pH	6.93 ±0.21	6.98 ±0.27	7.20 ±0.12

[1] Biodegradability is always 2% higher than biomethanization in all digesters due to the COD degraded by the sulphate reducing bacteria (SRB) that has been included as biodegradable COD but obviously, this COD degradation did not increase methane production. SRB presence is due to the sulphate concentration in the influent (see Table 2).

3.2.2. Effect of temperature

To assess the effect of temperature on microalgae biodegradability, a thermophilic CSTR was operated under the same experimental conditions that those established in mesophilic CSTR (Table 1) and was fed with the same influent (Table 2). Figure 2 shows the time course evolution of the thermophilic reactor in terms of: biogas flow rate and methane percentage in the biogas, COD in the influent, in the reactor and COD removal percentage, ammonium in the influent and in the reactor and free ammonia in the system, volatile fatty acids and alkalinity in the reactor, and reactor suspended solids concentration. As can be seen in Figure 2a, the biogas production achieved values around 7.05 mL· gVSS$_{reactor}$$^{-1}$ with a constant percentage of methane content of 70.0%, which results in 30.4% of biodegradability and 105.4 mLCH$_4$·gCOD$_{inf}$ $^{-1}$ of methane yield (Table 3). CSTR under thermophilic conditions increased methane yield 3-fold compared with mesophilic CSTR. Once pseudo-steady state was achieved (around day 90), COD removal achieved 36.0% (Figure 2b), reaching 5667 mg·L^{-1} of suspended solids in the reactor (Figure 2c). Similar trend was observed by Zamalloa et al. (2012a) who found that the biogas production rate was 1.3-fold higher at 55ºC than at 35ºC digesting *Scenedesmus obliquus* within a CSTR configuration.

The higher degradation observed in the thermophilic CSTR compared with mesophilic CSTR resulted in a higher ammonium concentration in the reactor, giving rise to a 63.7% of nitrogen mineralization. However, free ammonia concentration remained at similar values for the mesophilic CSTR (Figure 2d), around 20 mg·L^{-1}, being clearly lower than inhibition concentration level of 80 mg L^{-1} (Garcia and Angenent, 2009).

Moreover, stable volatile fatty acids and high alkalinity in the effluent confirmed that inhibition did not occur (Figure 2e). Table 3 shows a significant greater degradation operating a CSTR at 55ºC than at 35ºC. However, mesophilic AnMBR operated at 70 days of SRT and equal OLR exhibited the highest microalgal biomethanization due to higher SRT promoted microorganisms involved in anaerobic biodegradation of microalgal compounds such as cellulose, giving rise to a higher hydrolytic activity at mesophilic temperature (70 days SRT) than at thermophilic one (50 days SRT) as demonstrated below in microbial community analysis.

Since neither overload (stable effluent total volatile fatty acids –TVFA– concentration < 10 mg·L^{-1} in mesophilic digesters and < 200 mg·L^{-1} in the thermophilic) nor free ammonia inhibition occurred, the low anaerobic biodegradability of *Scenedesmus* microalgae observed can be attributed to its rigid cell wall as well as to the amount of slowly digestible organic residues after digestion.
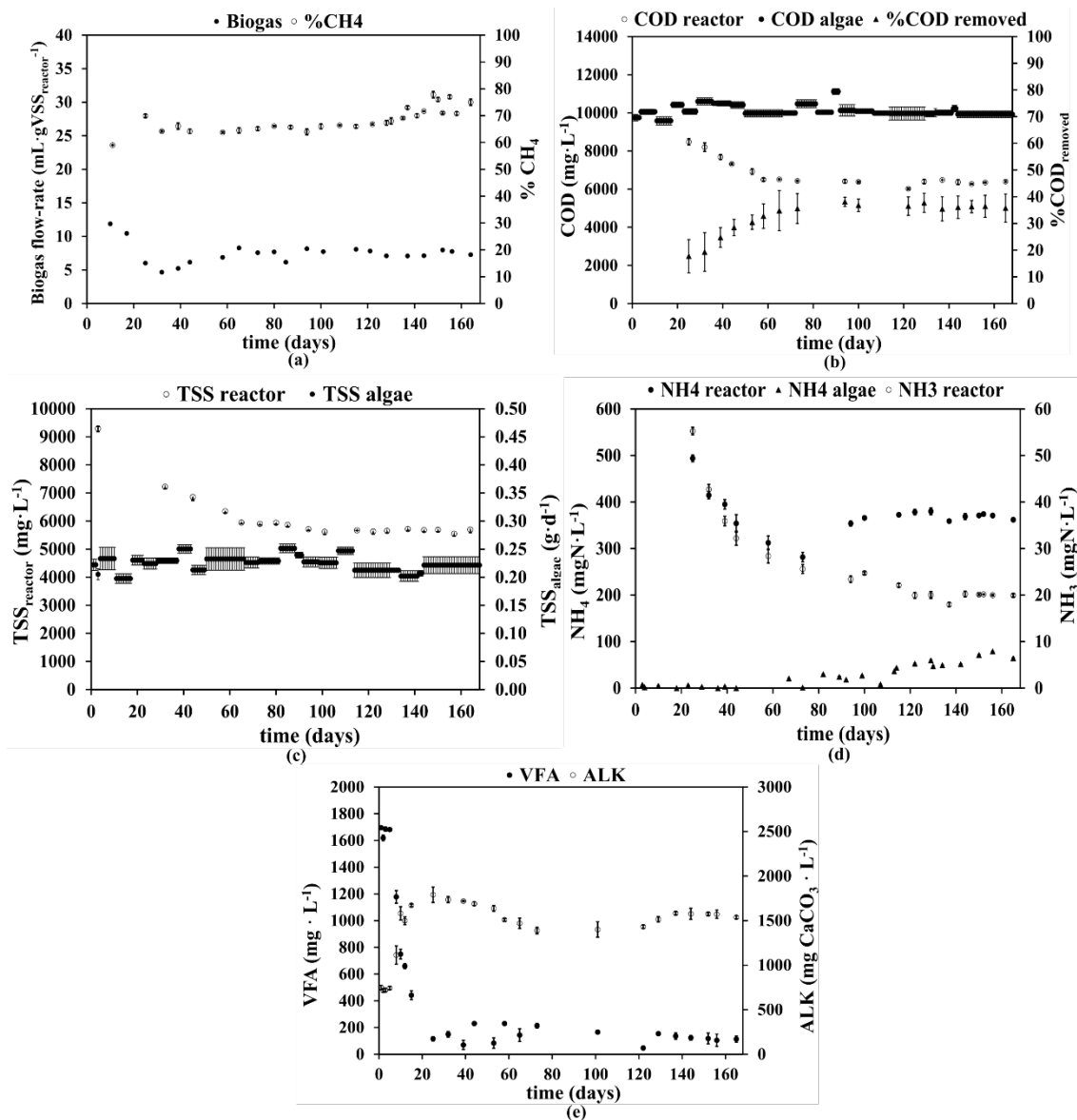

Figure 2. Temporal evolution along the experimental digestion in thermophilic conditions of: **(a)** biogas production per gram of VSS in the reactor and methane percentage **(b)** COD in the influent, in the reactor and COD removed percentage **(c)**

suspended solids in the influent and in the reactor **(d)** ammonia concentration in the influent and in the reactor and **(e)** volatile fatty acids and alkalinity in the reactor.

These results showed that the biodegradability of *Scenedesmus* fresh microalgae achieved higher values when the mesophilic reactor was operated at higher SRT and also when the reactor was operated at high temperature (55ºC). However, further studies are needed to maximize the energy recovery from microalgae biomass such as a thermophilic anaerobic digestion combined with membrane technology, since it was demonstrate the effectiveness of this technology in the present study. Likewise, the knowledge of the microbial communities present in both reactors, mesophilic AnMBR and thermophilic CSTR, could contribute to the optimization of the process since the distribution of the hydrolytic microorganism plays a key role in the microalgal biodegradation.

3.3. Microbial community analysis
Microbial population established in mesophilic and thermophilic digesters was studied when the highest biodegradability of *Scenedesmus* spp. was achieved in each reactor. The complexity of these systems and the poor microbial characterization of anaerobic digesters reported up to now, makes it necessary to broaden the knowledge of biological processes in order to detect microorganisms that are playing an important role in the improvement of microalgae degradation.

3.3.1. 16S rRNA gene amplicon sequencing
Under mesophilic operational conditions 96.8% of *genera*-based taxonomic units belonged to *Bacteria* and 3.2% to *Archaea* domains, whereas in thermophilic system these percentages were 97.4% and 2.6%, respectively. Results of relative abundance of different domain detected are shown in Figure 3. In this figure, taxonomic units represented at phylum or order level below 1.0% were grouped and plotted together as "other" *genera*-based taxonomic group.
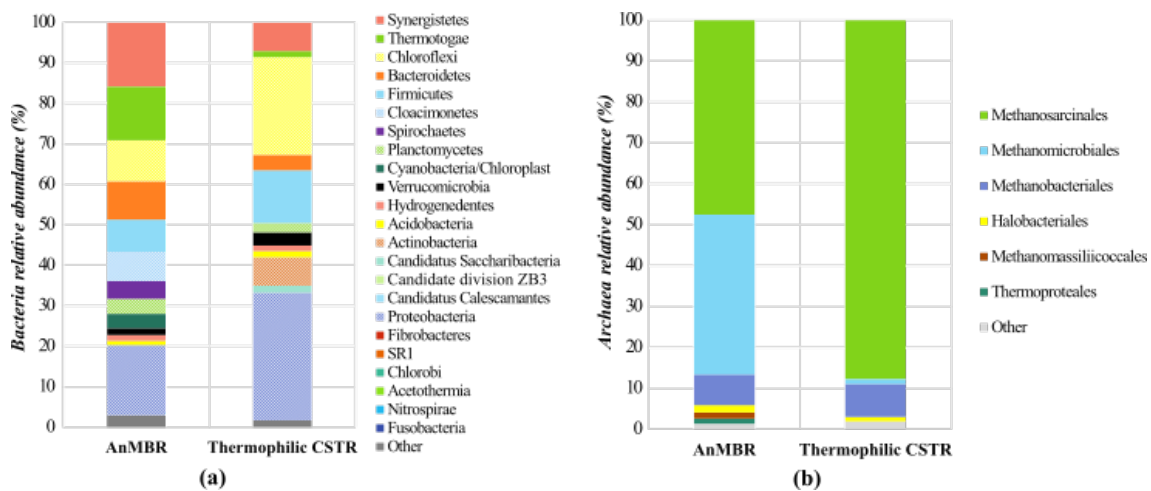

Figure 3. Relative abundance of **(a)** *Bacteria* genera-based taxonomic units **(b)** *Archaea* genera-based taxonomic units.

3.3.2. Archaea population

A noticeable group of known methanogenic *Archaea* orders were detected in both thermophilic and mesophilic reactors, 94.1% and 96.1% of the *Archaea genera*-based taxonomic units respectively (see Figure 3b). In thermophilic CSTR, *Methanosarcinales* were the dominant methanogens within a 87.0% of relative abundance, further followed by hydrogenotrophic and methylotrophic methanogenic orders like *Methanobacteriales* (8.0%) and *Methanomicrobiales* (1.2%). 80.7% of the *Archaea* sequences detected in this reactor corresponded to *Methanosaeta* genus (see Table S1), which commonly dominates anaerobic digesters, due to its capacity of releasing methane through acetoclastic pathways. Considering that these microorganisms are not only hydrogenotrophs, but also acetotrophs, acetoclastic seems to be an important methane releasing mechanism under thermophile conditions. Methanogenic population in AnMBR revealed that more than one methanogenic pathways could be followed, due to the order distribution observed: *Methanosarcinales* (47.6%), *Methanomicrobiales* (39.1%) and *Methanobacteriales* (7.5%). The higher diversity in mesophilic reactor obtained in this study, compared with thermophilic reactor, is in accordance with the observations of Franke-Whittle et al. (2014). The presence of more than one methanogenic pathways explains the high capacity of the mesophilic system to adapt to a wide range of substrates.

3.3.3. Bacteria population in mesophilic AnMBR
Six main phyla were identified in the AnMBR: *Proteobacteria* (17.3%), *Synergistetes* (15.9%), *Thermotogae* (13.2%), *Chloroflexi* (10.1%), *Bacteroidetes* (9.5%) and *Cloacimonetes* (7.2%) (Figure 3a). *Proteobacteria* is a heterogeneous phylum that includes microorganisms with different roles in the anaerobic digestion. In this case, a reasonable implication for this phylum would be the presence of sulphate-reducing bacteria (SRB) in the digester, due to the detected *Desulfobacterales* order, where most of the SRB are included (Plugge et al., 2011). Moreover, *Syntrophobacterales* are mostly composed of syntrophic microorganisms, like the identified genus *Smithella* (3.4% *Bacteria* relative abundance), capable of oxidizing a key intermediate compounds in anaerobic digestion such as propionate (Liu et al., 1999). Both orders belong to the *Deltaproteobacteria* class, which has a relative abundance of 38.4%. The relative abundance percentages of the genera belonging to the dominant *Bacteria* phyla are shown in Table S1.

The presence of *Thermotogae* in mesophilic digesters has been reported by other researchers (Nolla-Ardèvol et al., 2015; Sundberg et al., 2013), as they are well known dark fermenters and hydrogen producers (Pradhan et al., 2015). Inside this phylum it is included one of the most abundant genus found in the mesophilic AnMBR, *Fervidobacterium* (12.1% of *Bacteria*) which has been also observed when digesting cellulose substrates (Limam et al., 2014). Likewise, there is a remarkable abundance of *Synergistetes* phylum in this reactor that includes amino-acid degrading bacteria (Vartoukian et al., 2007), whose presence has been also observed when a high concentration of acetate was generated during mesophilic anaerobic digestion of a feedstock with high cellulose content (Li et al., 2014). *Cloacimonetes* and *Spirochaetes* phyla were detected only in AnMBR, as most of the known and belonging microorganisms related to these phyla seem to be mesophiles, and they have been detected in recent and similar studies (Li et al., 2014; Solli et al., 2014). Members of *Cloacimonetes* phylum are able to degrade compounds like amino-acids or cellulose (Limam et al., 2014), which are typical of microalgae feedstock.

3.3.4. Bacteria population in thermophilic CSTR

Proteobacteria was the most abundant *Bacteria* phylum identified in the thermophilic digester, with a relative abundance of 31.1% (Figure 3a). This phylum contains glucose fermenters and also acetate, butyrate and propionic degraders (Ariesyady et al., 2007). *Chloroflexi* phylum was also dominant in this reactor (23.8% of relative abundance) This phylum is involved in the degradation of polysaccharides such as cellulose (Hug et al., 2013).

Firmicutes (12.9%) is one of the most relevant phylum in anaerobic digesters, especially in thermophilic ones (Ritari et al., 2012). However, it was not dominant in this digester. Instead it has been overcome by *Proteobacteria* and *Chloroflexi* belonging microorganisms, widely extended in thermophile digesters. Other relevant phylum detected were *Synergistetes* (7.0%), also detected in mesophilic AnMBR. *Actinobacteria* (7.0%) seems to play another relevant role in the thermophilic reactor, due to their cellulose and xylan degrading capacity (Tuomela et al., 2000). *Thermotogae* (1.5%) abundance was not as high as reported in other studies of thermophilic sludge (Li et al., 2014).

3.3.5. Comparison of microalgae-degrading microbial community

As can be seen in Figure 3a, most of the dominant *Bacteria* phyla were detected in both digesters: *Firmicutes, Proteobacteria, Bacteroidetes, Chloroflexi, Thermotogae* and *Synergistetes.* In fact, these phyla have been reported to be able to degrade cellulose and proteins, which are found as a main components of microalgae cell-wall. In the present study, the changes in the relative abundance of each phylum under mesophilic conditions, compared with thermophilic conditions, are explained by the effect of the operational temperature since it is one of the most important selective factors (Li et al. 2014), even treating a complex substrate as microalgae feedstock. As it was found by Vanwonterghem et al. (2015), a low diversity is detected under thermophilic conditions during the anaerobic digestion of cellulose, which is in accordance with the lack of a dominant phylum in AnMBR digester where a more diverse microbial community was established compared with thermophilic reactor.

Furthermore, Wirth and co-workers (Wirth et al., 2015a) reported that anaerobic digestion of *Scenedesmus obliquus* within a mesophilic CSTR gave rise to a microbial community dominated by potential cellulose-degraders like *Firmicutes*. However, in the present study not only *Firmicutes* but also a wider community of cellulose-degraders with high hydrolytic potential was detected as a consequence of the high SRT through completely retention of the biomass by the membranes, which promote the establishment of low growth-rate microorganisms that might be positively correlated to high microalgae biodegradability. Therefore, the results obtained from the biological process supported by the conclusions from microbial analysis, propose the membrane technology as a suitable approach for microalgae anaerobic treatment.

Further studies are needed in order to understand the changes in microbial community, not only focusing on the operational temperature effects, but also on the operation at high SRT, which could act like a selective-pressure factor, conditioning the microbial population dynamics in digesters (Vanwonterghem et al., 2015). Thus, this microbial overview supported the feasibility of the total retention of biomass in the system by using AnMBR technology, which promoted microbial diversity when SRT was

increased, exhibiting a significant improvement in biogas production compared to conventional CSTR configuration and even compared to thermophilic conditions, resulting in a more robust microbial population less prone to process instabilities.

4. Conclusions

Anaerobic digestion of *Scenedesmus* spp. microalgae was studied under mesophilic conditions at two SRT within CSTR and AnMBR configuration, and also under thermophilic conditions within a CSTR. Regarding mesophilic digestion, AnMBR configuration increased methane production 4.2-fold compared to CSTR digester as a consequence of decoupling HRT and SRT, which allowed to operate at high SRT (70 days) and thus promoted low growth-rate microorganisms involved in microalgal degradation. Hence, microalgal biodegradability improved from 11.9% to 39.5%, which was higher than the result achieved in the thermophilic CSTR at 50 days of SRT (30.4%). These results suggest that anaerobic treatment using membrane technology under mesophilic conditions is able to achieve a great disruption of *Scenedesmus* cell-wall in order to revalue the microalgal biomass, even being the hydrolytic activity of the mesophilic degrader microorganisms lower than the microorganisms adapted to 55ºC. Regarding the microbial population, whereas at 55ºC microorganisms with high hydrolytic capacity were present (*Chloroflexi* and *Firmicutes*), more phyla cellulose-degraders (*Bacteroidetes*, *Chloroflexi*, *Firmicutes* and *Thermotogae*) were detected in AnMBR at 35ºC. Thus, membrane technology allowed to establish a more diverse community of cellulose-degraders, suggesting the presence of a more robust microbial population and that operating at higher SRT could significantly improve microalgae biodegradability.

In terms of nutrient solubilisation, nitrogen mineralization was greater than biodegradation levels, indicating the presence of slowly digestible organic residues with low nitrogen content after anaerobic digestion. Despite of the ammonium released from the disruption of protein content of microalgae, there was no inhibition of the anaerobic digestion process.

Appendix A. Supplementary data

The following is the supplementary data related to this article:

Figure S1. Schemes of lab-scale reactors: **(a)** mesophilic CSTR operated at 50 days of SRT, **(b)** mesophilic AnMBR operated at 70 days of SRT, **(c)** thermophilic CSTR ta 50 days of SRT, and their pictures: **(d)** and **(e)** respectively. Note that **(a)** and **(b)** correspond to the same lab-scale reactor (shown in **d**), which was first operated as CSTR and later converted into AnMBR with the incorporation of the membrane module.

Figure S2. Progression of transmembrane pressure during the operation of the mesophilic AnMBR.

Figure S3. Rarefaction curves obtained from AnMBR and Thermo-CSTR sequences retrieved from amplicon sequencing of 16S rRNA gene.

Table S1. Relative abundance percentages of the genera belonging to the dominant *Archaea* orders and *Bacteria* phyla detected in sludge samples sequenced, according to RDP's Classifier.

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