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

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Abstract

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

Keywords

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

1. Introduction

The composition of microalgae is heterogeneous among cultures, but it is commonly characterized by a high content in proteins and complex polysaccharides as cellulose-like layers [1]. Some *Chlorophyta* microalgae that can be used for nutrient removal like *Chlorella* and *Scenedesmus* genera have robust cell bodies [2]. Thus, their application of *Chlorophyta* for bioenergy production through anaerobic digestion can become challenging as this process is limited by the hydrolysis stage of the substrate [3]. Several authors have efficiently overpassed this barrier through the application of pre-treatments. According to Ometto *et al.* [4], enzymatic approaches exhibit the greatest biogas yield increments, compared to thermal or ultrasonication pre-treatment methods. However, these interesting strategies might not be feasible when upscaling the technologies for microalgae conversion into energy. As an alternative, other studies focus on the natural enzymatic capacity of several microorganisms for breaking recalcitrant plant-based compounds [5].

High biodegradation values of untreated microalgae had been previously reported from bioaugmentation processes with relevant cellulose degraders such as *Clostridium thermocellum* strain DSM 2360. The green microalgae *Chlorella vulgaris* was digested in a batch system with the consequent 24.0% increase of the methane yield [6]. A later study by Lavric and co-workers using the same strain achieved a 62.0% biodegradation of a microalgal mix from a high-rate algal pond, yet including a thermal pre-treating step for disruption [7]. These interesting strategies are microbial based and therefore

promising but lack relevant information about their viability in continuous systems like high retention anaerobic digesters.

The evaluation of the long-term stability of selected microbes and their enzymes in an engineered system should be explored to assess the feasibility of microbial based strategies [8]. According to this, a forward step for microalgae conversion into energy is required, *i.e*. new approaches should consider both the influence of operational conditions over microbial communities and their viability over time. Parameters like the Sludge Retention Time (SRT) or the Organic Loading Rate (OLR) have a strong influence over the diversity of microbial communities [9,10], and might affect the microalgae disruption efficiency. Acclimatized microbial communities must be not only suitable for raw microalgae disruption, but also resilient, tolerant or have functional redundancy to overcome process disturbances [11]. The main drawback of thermophilic digestion of protein enriched substrates such as microalgae is the inhibition by free ammonia [12]. Several microbial groups likewise the methanogens are sensitive to this reduced form of nitrogen, decreasing biomethanization yields in thermophilic reactors. Nevertheless, little is known about the effect of this inhibitor over the potential hydrolytic microbes and other key groups involved in the microalgae disruption. Although raw microalgae anaerobic digestion has been widely reported in mesophilic studies including continuous reactors [2,13], but with very little information about their microbial community compositions [14–16] thermophilic systems are less used for this purpose. However, operation temperatures over 50ºC might have a positive effect over hydrolytic microorganisms and their enzymatic reactions.

The present study evaluates the long-term community established in a thermophilic reactor for microalgae degradation. A raw microalgal biomass feedstock coming from a photobioreactor pilot plant was digested at high constant SRT and different OLR values

in a continuous system. A 16S rRNA gene analysis was performed along the 18-month experience revealing the composition of the biogas-producing thermophilic community for microalgae disruption established in the reactor.

2. Materials and Methods

2.1. Thermophilic reactor performance

Raw microalgae were continuously converted into biogas for 18-months in a thermophilic continuous stirred tank reactor of 1.6 L working volume (0.4 L headspace volume). The digester was inoculated with a thermophilic biomass coming from a pilot-scale digester (Valladolid, Spain). The biomass was mechanically stirred and maintained at 55ºC in the digester with an SRT and hydraulic retention time (HRT) of 50 days. The microalgae biomass was continuously harvested from a membrane photobioreactor pilot plant situated in the WTTP "Barranc del Carraixet" (Valencia, Spain) [17]. The phototrophic culture was dominated by *Scenedesmus* spp. according to González-Camejo *et al.* [18]. Microalgae were concentrated with a cross-flow ultrafiltration hollow-fibre system (5.7-11.7 $gVSS \cdot L^{-1}$). High free ammonia concentrations can be reached in anaerobic systems treating microalgae, especially under thermophilic conditions [12,19]. Thus, low OLR values (0.2, 0.3 and 0.4 $gCODinf \cdot L^{-1} \cdot d^{-1}$) were chosen in this study to avoid a process failure due to protein degradation (microalgae common content ranges 6-52% [12]), that can enhance free ammonia accumulation in the system. Three correspondent pseudo-steady state conditions were reached during each OLR scenario and characterized in terms of microbial population and main physico-chemical parameters. It was considered that the reactor run under pseudo-steady state conditions when the process exhibited stability in terms of solid concentration and biogas production for at least four weeks ($n \geq 4$).

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

This study is a long-term performance in a continuous reactor and therefore samples belonging to the same pseudo-steady state period are considered biological replicates of the thermophilic reactor microbial community. The samples were extracted from the reactor after 248, 268 and 276 days (samples T01, T02, T03; respectively from Period 1), 408, 422 and 443 days (samples T04, T05, T06; respectively from Period 2), 549 and 568 days (samples T07, T08; respectively from Period 3). Resulting pellets from 1 mL digestate samples were stored in 2 mL cryotubes at -20ºC. The E.Z.N.A DNA Extraction Kit for Soil (Omega-Biotek) was used for nucleic acid material extraction from 0.5 g biomass, according to the manufacturer's protocol. Resulting DNA was quantified in a fluorometric assay for dsDNA with Qubit 2.0 (Thermo Scientific).

16S rRNA gene analysis of *Bacteria* and *Archaea* microorganisms was performed through amplicon sequencing. Libraries were prepared using specific primers for the v3-4 hyper variable region of the target gene (341F 5'-CCTACGGGNGGCWGCAG-3' and 806R 5'-GGACTACNVGGGTWTCTAAT-3') [20]. The sequencing run was carried out in a 2x300 bp paired-end run using v3 chemistry in a MiSeq Sequencer (FISABIO, Valencia, Spain). Raw sequences were deposited on the NCBI database (BioProject PRJNA434206, SRP132920).

2.3.Illumina data processing

The resulting raw sequences were first trimmed and processed through the algorithm prinseq [21], applying a quality threshold of 30. The merging of each forward and reverse read was performed within fastq-join [22] and checked for chimeras with UCHIME [23] using default parameters. The downstream analysis of the filtered and high-quality resulting sequences was performed in QIIME 1. A 3% dissimilarity value between sequences was chosen for open reference otu-picking. The resulting Operational Taxonomic Units ($OTU_{0.97}$) were taxonomically assigned with the 16S rRNA-based LTP

128 release of the SILVA database. Final data was normalized to the minimum number of filtered paired-end sequences obtained. Additionally, $OTU_{0.97}$ below 0.01% were removed to reduce biases, as Lê Cao *et al.* [24] proposed for statistical analysis of amplicon sequencing data. Those $OTU_{0.97}$ assigned to *Cyanobacteria/Chloroplast* were attributed to the feedstock and non-functional organelles or cell bodies and thus not considered for microbial ecology analysis of digester.

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

Biodiversity of the thermophilic community was evaluated through the estimator *Simpson* index, which accounts alpha diversity considering species richness. Beta diversity of the community was explored through Principal Co-ordinate Analysis of the weighted UniFrac distance matrix retrieved from QIIME. Partial Least Square-Discriminant Analysis (PLS-DA) was performed in R-Studio according to Lê Cao *et al.* [24] to analyse the free ammonia effect over the microbial community members. PLS-DA is a powerful statistical modeling approach that allow the interpretation of big data matrixes like those resulted from the thermophilic reactor microbial composition 16S rRNA gene analysis. This multivariate statistical technique is usually performed to sharpen and maximize the separation between groups of samples according to their covariance values. PLS-DA is very useful because provides invaluable insight into the causes of the discrimination through the weights and loadings of the constructed model. The most discriminant groups were extracted after PLS-DA according to their Variable Importance Parameter (VIP).

3. Results and discussion

3.1.Microalgae biomethanization during thermophilic reactor performance

Three stable periods defined by an OLR of 0.2, 0.3 and 0.4 $gCODinf \cdot L^{-1} \cdot d^{-1}$ were achieved in the reactor using raw microalgae biomass as the only feedstock. The parameters determined thrice weekly during the three different pseudo-steady state periods are summarized in Table 1, including the mean values and the standard deviation of CH_4 percentage content in biogas, methane yield (calculated as $mLCH_4.gCOD_{inf}^{-1}$), concentration of ammonia ($mgN\text{-}NH_4 \cdot L^{-1}$), free ammonia concentration ($mgN\text{-}NH_3 \cdot L^{-1}$), VFA concentration ($mgCH_3COOH \cdot L^{-1}$) and alkalinity (measured as $mgCaCO_3 \cdot L^{-1}$). The complete performance of this reactor is well reported in a recent work [25].

Table 1. Characterization of the thermophilic reactor periods used for microbial analysis, mean values and standard deviation are shown (n≥4).

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

*Calculated considering a theoretical potential of 350 $mLCH_4 \cdot gCOD_{influent}^{-1}$ [25].

The anaerobic biodegradability of Scenedesmus biomass ranges 22-24% under mesophilic conditions, according to Gonzalez-Fernandez et al [25,26]. A recent study [15] performed under thermophilic conditions using an acclimated inocula reached a methane yield of 108.2 mL $CH_4 \cdot gCOD_{influent}^{-1}$ from raw Scenedesmus biomass. Hence, the favourable effect of acclimation of the microbial population in this work is here suggested. Biodegradabilities around 32-41% were achieved in the thermophilic CSTR operated at high solids retention time. The higher acclimation of the biomass to the microalgae feedstock, the higher release of hydrolysed compounds that can be finally turned out into methanogenic substrates. However, the low C:N ratio of this biomass might have an effect over the methanogenic activity. Indeed, the limited methane yield observed during Period 2 might be related to the presence of free ammonia forms that could inhibit the methanogenic population. Besides, the high concentration of the feedstock (5.7-11.7 gVSS·L-1) applied to maintain both HRT and SRT at 50 days could disturb the biological process.

3.2.16S rRNA sequencing data processing results.

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

diversity analysis of each sample reported valuable information about the number of species detected through open reference $OTU_{0.97}$ clustering analysis. A considerably high diversity in terms of species richness was elucidated from the more than 1,500 $OTU_{0.97}$ found during the whole experience (Table 2). Environmental samples are typically inhabited by complex communities characterized by a high number of observed species [11]. The number of observed species in the reactor ranged 1,445-2,621 and consequently a high Simpson index was observed between samples (0.86±0.03, n≥2). These values are among the range of values observed in similar studies *e.g.* thermophilic reactor treating a complex polysaccharide substrate, 0.72-0.98 Simpson index [27].

Table 2. Amplicon sequencing approach related information and alpha diversity analysis among samples

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

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

Compared to an extended diversity survey over thermophilic and mesophilic full-scale digesters based on pyrosequencing of the 16S rRNA gene [28], the diversity characterizing the thermophilic microalgae reactor is high in terms of observed OTUs. This diversity was taxonomically assigned to 50 phyla, 139 classes, 232 orders, 410 families and 823 genera from both *Bacteria* and *Archaea* domains. The microbial community found in the reactor was mainly assigned to six dominant *Bacteria* phyla (*Thermotogae*, *Firmicutes*, *Atribacteria*, *Aminicenantes*, *Synergistetes* and *Planctomycetes*) and two *Archaea* orders (*Methanobacteriales* and *Methanosarcinales)*, *including a Methanosaeta* related $OTU_{0.97}$ detected by the open clustering approach. These phylotypes were dominant in the reactor and represented the 77.3±3.9% of the cumulative community relative abundance (see Table 3). According to several authors, high diversity in anaerobic digesters is linked to a good performance [9]. However, it

should be remarked that biodiversity *per se* cannot guarantee the stability of anaerobic systems. Ecological aspects such as functional redundancy, resilience and resistance [29] of a certain community should be evaluated to help us to improve process stability in anaerobic reactors though a deeper comprehension of microbial community composition.

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

The microalgae mixed culture harvested from the photobioreactor pilot plant was mainly composed of *Scenedesmus* spp, which are characterized by a thick cellulosic material layer and a recalcitrant biopolymer *i.e. algaenan*, plus a mixture of neutral sugars, proteins and uronic acid [1]. Thus, this *Chlorophyta* microalgae can trigger different degradation anaerobic pathways suitable for methane production. For microbial ecology interpretation, only methanogenic *Archaea* and those dominant *Bacteria* phylotypes defined at genus level with relative abundance values over 2.0% were selected. Figure 1 shows the composition of the different phylotypes found in each sample during the microalgae thermophilic digestion.

Table 3. Relative abundance values of dominant phylotypes detected in the thermophilic reactor.

			Period 1 (OLR = 0.2)			Period 2 (OLR = 0.4)			Period 3 (OLR = 0.3)		
SILVA Accession No.	**Role***	**Ref.***	**T01**	**T02**	**T03**	**T04**	**T05**	**T06**	**T07**	**T08**	**Taxonomic Classification****
EF515700.1.1413	PF	[30]	4.8	7.0	6.0	0.0	0.0	0.0	0.2	0.3	d.-Bacteria; p.-Aminicenantes
EF586052.1.1457	SF	[31]	2.6	1.6	1.8	2.4	2.7	2.5	3.4	2.9	d.-Bacteria; p.-Atribacteria; c.-Atribacteria Incertae Sedis; o.-Unknown Order; f.-Unknown Family; g.-Candidatus Caldatribacterium
CP001251.796084.797624	H,SF	[32]	2.2	3.9	3.0	6.9	4.9	4.0	3.8	4.4	d.-Bacteria; p.-Dictyoglomi; c.-Dictyoglomia; o.-Dictyoglomales; f.-Dictyoglomaceae; g.-Dictyoglomus spp.
FJ769444.1.1396	H,PF	[33]	35.6	32.8	33.0	19.7	18.4	15.1	19.7	14.9	d.-Bacteria; p.-Firmicutes; c.-Clostridia; o.-Thermoanaerobacterales; f.-Thermodesulfobiaceae; g.-Coprothermobacter sp.
FN436058.1.1503	SF	[34]	3.4	3.6	3.7	1.5	0.9	1.5	2.0	2.8	d.-Bacteria; p.-Planctomycetes; c.-Planctomycetacia; o.-Planctomycetales; f.-Planctomycetaceae; g.-Thermogutta sp.
EF559055.1.1480	PF	[27]	9.3	9.5	9.0	6.7	4.2	5.9	7.8	6.1	d.-Bacteria; p.-Synergistetes; c.-Synergistia; o.-Synergistales; f.-Synergistaceae; g.-Anaerobaculum sp.
AY862527.1.1347	-	[35–37]	8.0	2.8	11.7	37.6	40.7	36.4	39.7	38.9	d.-Bacteria; p.-Thermotogae; c.-Thermotogae; o.-EM3
CU919211.1.1345	PF	[38]	4.7	4.1	2.8	2.9	3.1	2.3	2.0	2.6	d.-Bacteria; p.-Thermotogae; c.-Thermotogae; o.-Petrotogales; f.-Petrotogaceae; g.-Defluviitoga sp.
EU638683.1.1349	H,SF	[5]	2.0	8.0	3.9	3.3	3.0	4.5	2.2	5.6	d.-Bacteria; p.-Thermotogae; c.-Thermotogae; o.-Thermotogales; f.-Fervidobacteriaceae; g.-Fervidobacterium sp.
DQ785500.1.922	HM	[28,38]	0.2	0.1	0.2	0.5	0.3	0.1	0.2	0.2	d.-Archaea; p.-Euryarchaeota; c.-Methanobacteria; o.-Methanobacteriales; f.-Methanobacteriaceae
FN646546.1.939	HM	[28,38]	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0	d.-Archaea; p.-Euryarchaeota; c.-Methanobacteria; o.-Methanobacteriales; f.-Methanobacteriaceae; g.- Methanothermobacter sp.
New ref. OTU#204	AM	[28,38]	0.5	0.8	0.7	1.4	0.6	0.3	0.5	0.4	d.-Archaea; p.-Euryarchaeota; c.-Methanomicrobia; o.-Methanosarcinales; f.-Methanosaetaceae; g.-Methanosaeta sp.
Cumulative relative abundance (%)			73.5	74.2	75.9	83.0	78.9	72.7	81.6	79.0	

*Role and reference used for ecological interpretation, H: hydrolytic; SF: saccharolytic fermenter; PF: peptidic fermenter; B: 2F: second fermenter; HM: hydrogenotrophic methanogen; AM: acetoclastic methanogen.

**Taxonomic levels have been abbreviated from domain (d.) to genus (g.) level.

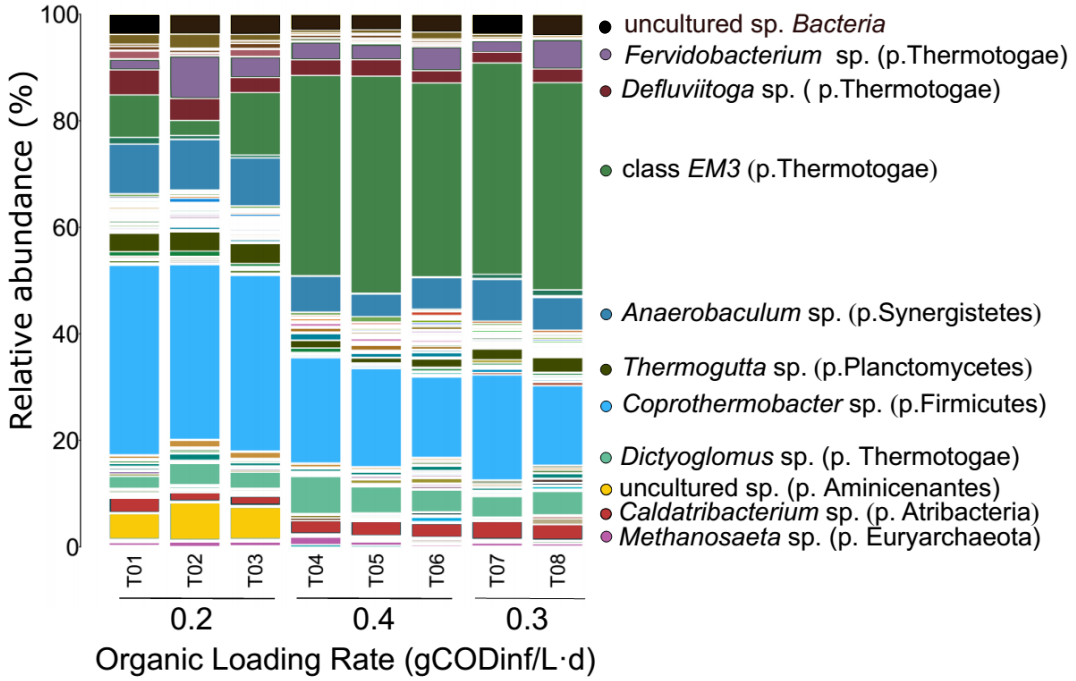

Figure 1. Relevant phylotypes classified at the lowest known taxonomic level among the three periods studied in the thermophilic reactor. White colour has been used for phylotypes below 2% relative abundance in every sample to facilitate the visualization of the barplots. Samples belonging to the same pseudo-steady state period are biological replicates of reactor biomass.

The significant content of proteins of *Scenedesmus* microalgae [1,19] makes peptidic hydrolytic and fermentative pathways during anaerobic digestion critical. *Coprothermobacter* was among the dominant phylotypes observed in the reactor. The overwhelming potential of *Coprothermobacter* related microorganisms for protein degradation is well known and has been extendedly reviewed [33]. Its relative abundance in the thermophilic reactor ranged among 15.1-35.6% during the continuous operation. These results suggest the implication of this phylotype in the first stage of the microalgae disruption by releasing peptidases that can degrade *Scenedesmus* cells.

After the disruption of proteins, amino acid fermenters like *Aminicenantes* and *Anaerobaculum* phylotypes can easily take the released peptides. No cultured strain has been related up to date inside the *Aminicenantes* phylum. However, as Kuroda and co-workers recently reported [30], amino acid fermentation might be their main implication in anaerobic digesters. *Anaerobaculum* related microorganisms have been previously

observed in thermophilic full-scale digesters treating plant-derived substances [39]. These authors also suggested the peptidic fermentation capacity of these microorganisms. Other fermenters detected in the present study such as *Caldatribacterium*, *Thermogutta* and *Fervidobacterium*, have been associated on the other hand to saccharolytic pathways [30,31,33]. The relevance of these groups in anaerobic digesters relies on their implication as methanogenic precursor producers, as acetate, carbon dioxide and hydrogen are among their fermentative products.

The remarkable relative abundance of hydrolytic and fermentative phylotypes in the thermophilic reactor was attributed to the capacity of the digester biomass for microalgae degradation. The presence of the phylotypes *Dictyoglomus*, *Defluviitoga* besides the abovementioned *Caldatribacterium*, *Thermogutta* and *Fervidobacterium* has been reported in studies where recalcitrant substrates were treated at high operational temperature. Several glycosyl hydrolases were found in a recent metagenomic study that revealed the important role of *Dictyoglomus* genus in the fermentation of plant-based carbohydrates [32]. The metatranscriptomic analysis of a full-scale thermophilic plant treating agricultural wastes highlighted the role of *Defluviitoga* for breaking hemi-celluloses [38], which are some of the main constituents of *Scenedesmus* cell walls. The authors found high relative activity values of *Defluviitoga* and suggested their role as main saccharolytic fermenters. A recent multi-omic study supports the relevance of the *Thermogutta* phylotype during fermentation stages in thermophilic digesters [34]. Little is known about the *Atribacteria* phylum and one of the phylotypes identified in the present study: *Caldatribacterium*. According to a recent metagenomic reconstruction from different environmental samples including anaerobic digesters [40], this phylotype is a potential carbohydrate fermenter. In this thermophilic microalgae digester, the higher hydrolysis of microalgae cell walls, the higher release of cellulose- and hemicellulose-

derived monomers that might trigger the presence of saccharolytic fermenters. Finally, the remarkable presence of the EM3 group in the reactor must be separately discussed as its metabolic roles remain poorly characterized (to the knowledge of the authors of the present manuscript).

This is the first study of microbial dynamics in an anaerobic digester where high relative abundance values of EM3 group have been continuously observed. This group was recently affiliated to the *Thermotoga* phyla after metagenomic analysis of the anoxic under layer of phototrophic microbial mats [35]. According to these authors, EM3 related microorganisms could be involved in fermentation pathways, providing hydrogen to other members of the microbial community. Nevertheless, other previously reported studies suggested that the metabolic implications of EM3 in a thermophilic digester could be more diverse, as its presence has been detected in a similar system treating lignocellulose although in lower relative abundances compared to the present study [36]. On the other hand, a previous study based on RNA stable isotope probing identified EM3-related microbial groups during the thermophilic conversion of long-chain fatty acids into methane [37]. Hydrogen is commonly released during fermentation of hydrolysed microalgae components under thermophilic conditions. Also, beta oxidation of long-chain fatty acids should be considered when treating microalgae grown in a pilot plant, as stress conditions boost the lipid intracellular accumulation in the microalgae bodies [41]. Hence, suggested metabolic roles for EM3 group in this study would be: (i) disruption of microalgae cell walls, (ii) uptake of substrates released after microalgae hydrolysis such as carbohydrate monomers or (iii) beta oxidation of intracellular lipids. Further research with a deeper comprehension of the metabolic pathways of EM3 could help to elucidate the role of this group in degradation of *Scenedesmus* biomass.

Summarizing, the coexistence of potential proteolytic phylotypes such as *Coprothermobacter*, jointly with scavengers of amino acids like *Anaerobaculum*; besides the suitable hydrolytic and saccharolytic role of *Fervidobacterium*, *Dictyoglomi*, and *Defluviitoga* phylotypes and the potential implication of EM3 in other relevant pathways (hydrolysis, hydrogen-producing fermentation or beta-oxidation), might allow the disruption of untreated microalgae into different by-products that can be further turned into methane by methanogenic microorganisms. Furthermore, the syntrophic relationships among these dominant microbes might have also allowed the thermophilic reactor to host a fast transference of the even more reduced compounds released from the digested microalgae cell bodies. The presence of these relevant phylotypes over time in the reactor operated at high SRT (50 days) suggests their convenient use for continuous degradation of raw microalgae.

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

An appropriate environment for methane production was promoted in the reactor since microalgae degradation releases methane precursors such as acetate as well as carbon dioxide and hydrogen, giving rise to the methane productivities shown in Table 1.

In the present study, according to the relative abundances observed of methanogenic phyotypes, the dominant group were *Methanosaeta* spp. (Table 3). The acetoclastic pathway is well reported for this group of methanogens [28,38] and several authors have suggested its acclimatization capacity to free ammonia for enhancing the digester performance in terms of stability and methane production when degrading microalgae [42]. However, this group dramatically decreased during Period 2 from 1.4% to 0.3% (Table 3). It should be remarked that the highest concentration of *Scenedesmus* was fed to the reactor during this period, where also a VFA accumulation of 491.1

mg$CH_3COOH \cdot L^{-1}$ was determined. These results suggest that the highest hydrolytic activity of the thermophilic reactor was achieved in Period 2 but did not result in highest biomethanization values due to a partial inhibition of the methanogenic pathways.

Only minor groups of hydrogenotrophic methanogens belonging to *Methanobacteriaceae* were observed besides *Methanosaeta* in the thermophilic reactor, suggesting that the acetoclastic pathways were dominant in the reactor. The relative abundance of these methanogens increased 2-fold during Period 2, where the maximum OLR of *Scenedesmus* was fed to the reactor. As abovementioned, this period was characterized by a VFA accumulation and suggested as a partially inhibited methanogenic state. Hydrogen scavenging by *Methanobacteriaceae* has a relevant role in thermophilic digesters or when acetoclastic methanogens are inhibited, providing robustness and resilience to the process [34]. In the same logic, the microalgae digester might recover its methanogenic capacity in Period 3 after enhancing other methanogenic pathways different to the acetoclastic, as an absence of VFA accumulation and the highest biomethanization values 143.7±3.9 m$LCH_4 \cdot gCOD_{inf}^{-1}$ were finally observed in Period 3. In this period, *Methanosaeta* remained as dominant methanogens although at lower relative abundances (0.4±0.1%) than in previous periods. These findings suggest a slight acclimatization capacity of *Methanosaeta* to the free ammonia values achieved in the thermophilic digester (62.23±3.09 mgN-$NH_3 \cdot L^{-1}$).

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

The disruption of the microalgae cell bodies was carried out by the presumably hydrolytic population enriched during long-term operation of the reactor. According to different studies feedstock has a strong influence over the microbial community structure of anaerobic reactors [10,11]. In the present study, the characteristics of *Scenedesmus*

biomass might shape the microbial community over-time. This hypothesis was explored by weighted UniFrac phylogenetic distances calculation between samples and later principal component analysis of the resulting matrix (see the Principal Co-ordinates Analysis (PCoA) resulting plot in Figure 2).

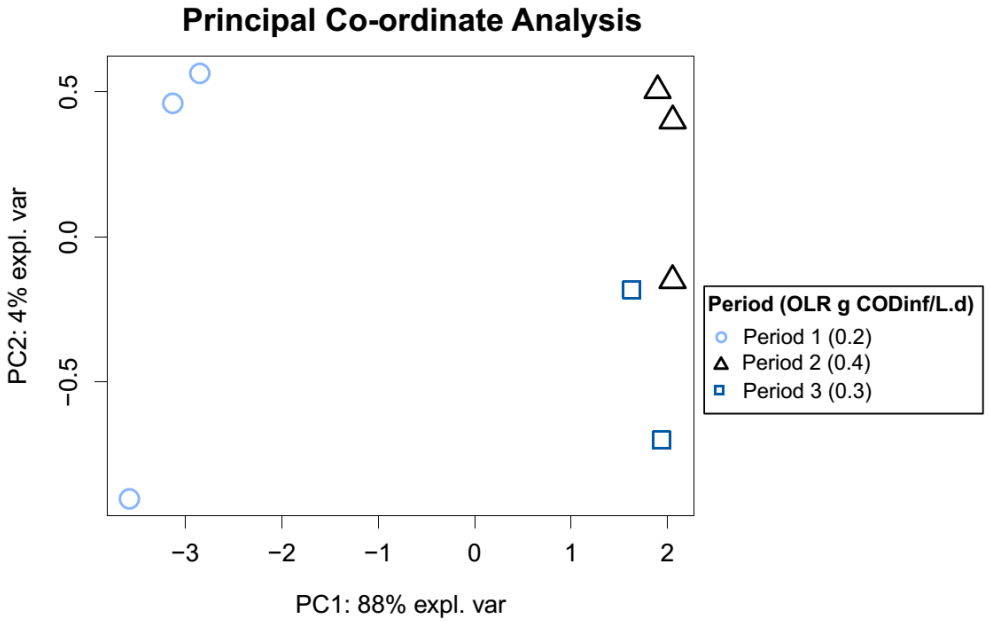

Figure 2. Principal Co-ordinates Analysis (PCoA) of weighted UniFrac thermophilic $OTU_{0.97}$ distance matrix. Explained variance by each component is indicated in each axis in percentages.

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

The resilience of the community established during Period 2 can be suggested from the results observed in Figure 3. As shown in Table 1, a partial inhibition of the methanogenic members of the thermophilic reactor was elucidated from the consequent VFA accumulation in the reactor. However, the second component extracted from the PCoA has a very low explanatory value of 4%. Although the main difference in between Period 2 and 3 was the presence of absence of VFA accumulation due to the free ammonia values achieved, the microbial community structure of the thermophilic reactor remained stable. Hence, the presumably hydrolytic bacteria members found in the present study for *Scenedesmus* disruption in absence of other pre-treatments would have a certain tolerance to free ammonia concentrations that on the other hand affected the activity of other microorganisms like *Methanosaeta*.

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

The shift in the microbial diversity observed during VFA accumulation after partial inhibition by free ammonia released in the reactor has been thoroughly discussed in this study through analysis of the most abundant phylotypes. However, less abundant groups might also have a relevant response in the digester to operational disturbances. From an ecological perspective, the presence of minor groups can provide resilience to a certain environment when they are functionally redundant as different microorganisms inhabiting the same niche can be involved in similar metabolic pathways but have different phylogenetic or physiological characteristics [43].

PLS-DA analysis was applied to the microbial community composition discriminating between the presence of potential inhibitors like free ammonia observed at low (Periods 1 and 3) or high (Period 2) concentration in the thermophilic reactor. This inhibitor can act as selective factor decreasing the diversity of the system, but it might

also enhance the resilience of the biomass, as some of the key microorganisms might have acclimatization potential to inhibitors. As can be seen in Figure 3, the developed PLS-DA model extracted for free ammonia perfectly separates the observations from low and high inhibition levels reached in the reactor.

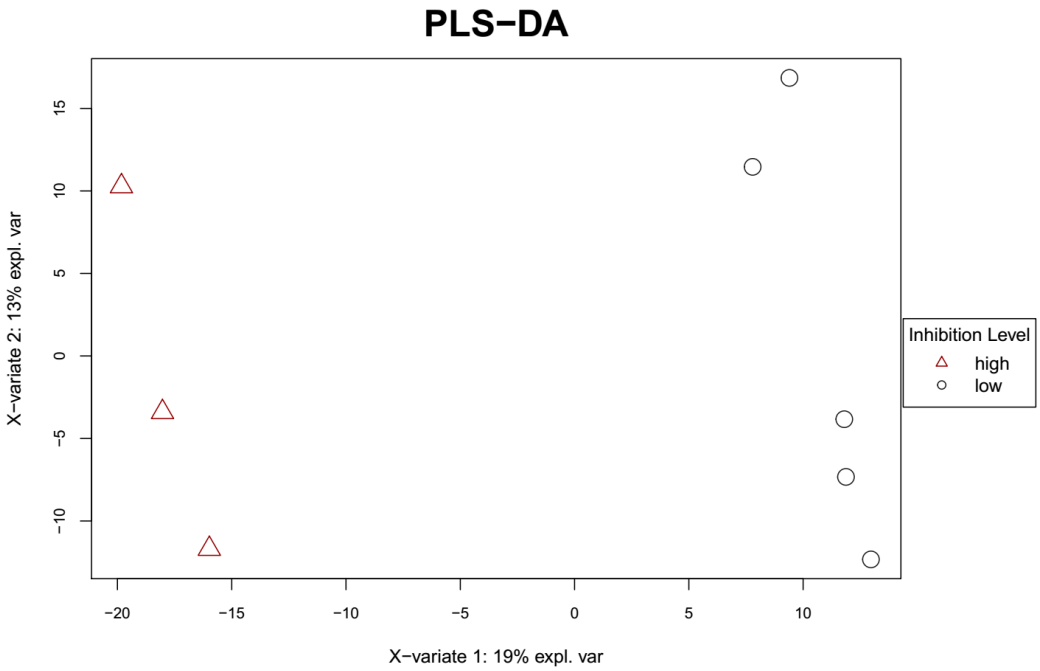

Figure 3. Evaluation of the high and low inhibitor level effect over reactor samples through Partial Least Squares Discriminant Analysis (PLS-DA). Samples with high levels of inhibitors (Period 2) are plotted with red triangles and low levels (Periods 1 and 3) with circle points.

The resulting PLS-DA plot shown in Figure 3 was constructed from the first and the second components extracted from the analysis, which showed up the highest variability values. The first component explained a 19% of the covariance among samples, whereas the second component denoted a 13% (related to the feedstock influence as previously discussed in this manuscript). To reveal the most discriminant microbial groups fitting the model, the Variable Importance in the Projection coefficient (VIP) was extracted from the PLS-DA. VIP values are indicators of the explanatory power of each $OTU_{0.97}$ over the predicted variate (high level inhibitors, in this case). The discriminant $OTU_{0.97}$ values were decreasingly sorted according to their VIP value. The 30 top $OTU_{0.97}$ groups are shown in Table 4 in alphabetical order of their taxonomy for ecological interpretation.

Table 4. Variable Importance in the Projection (VIP) of each $OTU_{0.97}$ from PLS-DA analysis

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

Phylotypes affiliated to *Ruminococcaceae*, *Thermogutta* and *Armatimonadetes* were among the most discriminant (Table 4) during the accumulation of VFA in the digester in Period 2 because of the partial inhibition by free ammonia (see Table 1). Microorganisms belonging to the *Ruminococcaceae* family have been found during fermentation and biohydrogenation of *Scenedesmus* biomass [44]. Besides, a recent multi-omic study supported the relevance of the *Thermogutta* genus during fermentation stages in thermophilic digesters despite their common low abundance [34]. Finally, related groups to the *Armatimonadetes* phylum contain several species with a strong capacity for branched or amorphous polysaccharide disruption such as *Chthonomonas* [45]. Their prevalence in plant-fed digesters was suggested by Dunfield and co-workers [46]. Due to the lack of cultured strains from this phylum, it is still poorly characterized. Despite the low abundance of these minor phylotypes (as their relative abundances were below 2% and therefore not shown in Figure 1), their high explanatory power in the PLS-DA model extracted from the thermophilic 16S rRNA gene analysis suggests their relevance in the efficient conversion of raw microalgae into biogas in a continuous system. These results are in accordance with the hypothesis of functional redundancy in anaerobic digesters which has been suggested as an ecological strategy that ensures a reservoir of responses against different disturbances over time and thus stabilizing the system performance [11].

According to the results here presented, the disruption of microalgae by different dominant and minor bacteria groups with functional redundancy for *Scenedesmus* disruption allow the thermophilic conversion of this substrate into methane over time in a continuous system. Long-term acclimatization of methanogens to the free ammonia that is commonly released from a low C:N substrate like *Scenedesmus* should be considered in future studies as a microbial-based strategy to guarantee the performance of a

biological thermophilic anaerobic conversion of this microalgae. Also, the risk of the inhibition by free ammonia observed in the present study could be mitigated changing the low C:N ratio of the influent. As a future step forward, the addition of complementary substrates with high carbon content could be explored to benefit from the hydrolytic potential of the acclimated community without disturbing methanogenic population. Moreover, the addition of a co-substrate would allow to evaluate the feasibility of applying of this process at pilot or industrial scale, since full-scale digesters are operated at higher OLR values than the range chosen in the present work. All these concepts should be preferably explored in anaerobic reactors where hydraulic and solids retention time can be separately optimized, like the anaerobic membrane bioreactor (AnMBR) or the up-flow sludge blanket (UASB) systems. In this manner, hydraulic time could be modified and decreased to mitigate the inhibitive effect of free ammonia, facilitating the acclimation of methanogens to this compound. Finally, the combination of microalgae and other substrates resulting from municipalities (like food waste or sewage sludge streams) for bioenergy generation would be an attractive practice that would also meet with the basics of a circular economy perspective.

4. Conclusions

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

knowledge of microbial communities and allows the selection of future microbial groups that can be applied during biological conversion of complex microalgae in conventional systems.

Author Contributions

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

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Statements regarding conflicts, consent and human/animal rights

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

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