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Title: Biogas production from sewage sludge and microalgae co-digestion under mesophilic and thermophilic conditions

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Abstract: Isochrysis galbana and Selenastrum capricornutum, marine and freshwater species respectively, were co-digested with sewage sludge under mesophilic and thermophilic conditions. The substrates and the temperatures changed biogas production significantly.
Under mesophilic conditions, sewage sludge digestion produced 451+/-12 mLBiogas/gSV. Similarly, all digesters fed with Isochrysis galbana or the microalgae mixed with sludge averaged 440+/-25 mLBiogas/gSV. On the contrary, Selenastrum capricornutum, produced 271+/-6 mLBiogas/gSV and the mixtures with sludge produced intermediate values between sludge and microalgae production. The higher proportion of freshwater microalgae was, the lower the biogas yield was.
Under thermophilic conditions sewage sludge achieved again the highest biogas yield, 566+/-5 mLBiogas/gSV. During co-digestion, biogas production decreased when microalgae content increased; and Isochrysis galbana and Selenastrum capricornutum reached minimum values, 261+/-11 and 185+/-7 mLBiogas/gSV, respectively. However, no evidences of inhibition were found and the low yields were attributed to microalgae species characteristics.
Methane content in biogas showed similar values independently the digested substrate, although it increases approximately by 5% under thermophilic condition.

Cover Letter

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Tarragona, September 8th, 2014

Professor Soteris Kalogirou

Editor-in-Chief

Renewable Energy

Dear Professor Kalogirou,

We are pleased to submit a revision of our manuscript entitled:
"Biogas production from sewage sludge and microalgae co-digestion under mesophilic and thermophilic conditions";
to be considered for publication in Renewable Energy.

The manuscript was fully revised following comments of both reviewers. The quality of the manuscript was highly improved by the remarks of reviewers.

Finally, the language of the manuscript was checked by someone who speaks English as a first language.

Looking forward to hearing from you,

Sincerely yours,

Sincerely yours,

Dr. Christophe Bengoa
Corresponding author

Answers to the reviewer #1 comments.

The authors report on very interesting results of the anaerobic digestability and biogas yields of two algae species under mesophillic as well as thermophillic conditions. There are few points yet unclear: In the materials and methods section it is stated that the algae were cultured in Walne's medium prepared from sea water. Is it true that the fresh water species were also cultured in sea water?

The differences in digestabiliy are due to cell wall composition. Is the cell wall composition typical for sea water species compared to fresh water species or tyical for each species indepent of sea water or fresh water?

The language should revised thoroughly. In some cases it is difficult to follow because of poor English.

First of all, we would like to thank the reviewer's contribution, that allows to improve the manuscript. Following, you can find the changes carried out in connection with the comments of reviewer #1. The corrections from reviewer #1 are marked in blue in the revised manuscript.

1) "In the materials and methods section it is stated that the algae were cultured in Walne's medium prepared from sea water. Is it true that the fresh water species were also cultured in sea water?"

Answer: As the reviewer well said, it was stated in Section 2.1.2. that both microalgae species were cultured in Walne's medium, prepared with seawater. This statement is a mistake that has been corrected. Only the seawater species was cultured in this medium prepared with seawater. For the freshwater species cultivation, Woods Hole MBL medium was prepared, in this case, with deionised water. The sentence in lines 104-106 is modified by the sentences:

"For the marine species cultivation, Walne's medium [18] was prepared with filtered (0.45 µm), autoclaved seawater from Alfacs Bay (Ebro Delta, Spain). For the freshwater species cultivation, Woods Hole MBL medium [19] was prepared with deionised water, and autoclaved before inoculation."

On the other hand, the new Reference [19] was added and a renumbering of references was performed.

2) "The differences in digestabiliy are due to cell wall composition. Is the cell wall composition typical for sea water species compared to fresh water species or tyical for each species indepent of sea water or fresh water?"

Answer: As many authors conclude, differences in digestibility can be attributed to the presence or lack of cell walls in microalgae, and to the cell wall characteristics. Generally, the cell wall composition is typical of the microalgae species and also of algae classes; however, it may exists variations. For this reason, the characteristics of the cell walls are described.

In our experiments, only two species were used, saltwater and freshwater species. However, our results are compared with the work done by Mussgnug et al., where several microalgae species cultured in saltwater and freshwater were anaerobically digested [11]. The differences in biogas production were attributed to differences in cell wall characteristics. Species without cell wall were easily digested; when a cell wall was present, species with a protein-based cell wall were better digested than species with a carbohydrate-based cell wall.

As explained in section 3.1., the saltwater species *I. galbana* used in our experiments has not cell wall, being the reason of the high biogas production. For example, our result is comparable with the saltwater species *Dunaliella salina*, which also has not cell wall [11]. However, the saltwater species *Arthrosira platensis* produced a similar quantity of biogas, although their protein-based cell walls [11].

In regards to the freshwater species *S. capricornutum*, the low digestibility is comparable to other microalgae species with a carbohydrate-based. Mussgnug et al. observed that low biogas productions were obtained from *Scenedesmus obliquus* and *Chlorella kessleri*, both species with carbohydrate-based cell walls [11].

The sentences in lines 250-254 are modified by:

Sentences: "Additionally, it is fairly well known that the microalgae cell wall determines the potential conversion into biogas; *I. galbana* cells have been described as small size cells, with a lack of a cell wall which facilitates anaerobic digestion [11,31]. Furthermore, the change from a high saline to a non saline environment favours microalgae disintegration [30]."

The sentences in lines 275-279 are modified by:

Sentences: "*S. capricornutum* cell walls are thick and rigid carbohydrate-based structures, which act as strong barriers for microorganism reducing cell degradation [34]. Additionally, the carbohydrate-based cell wall species can resist degradation better than protein-based cell wall microalgae or microalgae without a cell wall [11]."

Answers to the reviewer #2 comments.

This paper covers the digestion of two types of algae that were grown on sewage sludge. It is a useful paper, although the English language needs to be improved. The process of using AD to recover energy from algae is a subject that is worthy of study. Algae can be grown on sewage sludge as it provide the required nutrients. However, AD can be used to process sewage sludge directly. It would have been useful to compare the amount of biogas obtained from a sample of sewage sludge directly with that obtained from the algae, but that would require further experimentation. This might be the subject of a later paper.

Firstly, we would like to thank the second reviewer's comments. The manuscript was entirely revised to avoid bad English usage and some paragraphs were rewritten for better comprehension. Following, you will find all the changes carried out in connection with the comments of reviewer #2. The corrections from reviewer #2 are marked in green in the revised manuscript.

1) "It would have been useful to compare the amount of biogas obtained from a sample of sewage sludge directly with that obtained from the algae, but that would require further experimentation."

Answer: It is true that the way the results are presented does not allow the reader to well identify the comparison between biogas production from microalgae digestion and from sewage sludge digestion. Some paragraphs were either modified or rewritten, and the order of some sentences was simply modified in order to clarify ideas and facilitate reader comprehension. This was done in both sections, 3.1. and 3.2., and for both microalgae species.

The paragraph between lines 209-224 is modified by:

Paragraph: "As it can be seen in Figure 1, the biogas production between reactors did not differ significantly, either when microalgae and sludge were digested separately or mixed. After 35 days, *I. galbana* digestion produced 439±4 mL_{Biogas}/g_{VS}. This value is higher than the reported for other saltwater microalgae like *Spirulina maxima* (330 mL_{Biogas}/g_{VS}) and for *Gracilaria* species and strains (280 and 400 mL_{Biogas}/g_{VS} respectively) [28,29]. In the same experiment, sewage sludge digestion produced 451±12 $mL_{Biogas}/g_{VS.}$; this value is in agreement with the values reported for sludge anaerobic digestion [1]. The comparison of microalgae and sewage sludge digestion highlights that microalgae are able to produce more or less the same quantity of biogas than sewage sludge, a substrate currently used in anaerobic digestion. In particular, *I. galbana* produces the same amount of biogas, demonstrating that this microalgae species is an eligible substrate for anaerobic digestion, competing with sewage sludge. This fact is confirmed by the results obtained during the anaerobic digestion of *Phaeodactylum tricornutum* in continuous mode (530 mL_{Biogas}/g_{VS}) and during the anaerobic digestion of *Arthrosira platensis* and *Dunaliella salina* in batch digesters (480 and 505 mL_{Biogas}/g_{VS} for respectively) [11,29]. The authors suggested that anaerobic digestion constitutes the best option to recover energy from these microalgae species.

The paragraph between lines 255-259 is modified by:

Paragraph: "Barely 271±6 mL_{Biogas}/g_{VS} were produced during the experiments with *S. capricornutum*; this production is the lowest obtained under mesophilic conditions (Figure 2). Although higher biogas productions were reported for freshwater microalgae species, similar results were observed in species characterised by carbohydrate-based cell walls, like in *S. capricornutum* [34]. Mussgnug et al. digested different microalgae species and observed that the species *Scenedesmus obliquus* and *Chlorella kessleri*, both with carbohydrate-based cell walls, showed lower biogas production and higher amount of indigestible residues than species without cell wall or with protein-based cell walls digested in the same conditions [11]. Even a lower biogas production was reported during *Scenedesmus sp*. digestion, only 75 mL_{Biogas}/g_{VS} after 35 days at mesophilic temperatures [32]. On the other side, comparison with biogas production from sewage sludge, digested in the same experiment, shows that *S. capricornutum* reached around 60% of the biogas production from sewage sludge, 451±12 $mL_{Biogas}/g_{VS.}$. This result differs from

the saltwater species *I. galbana*, which produced more or less the same quantity of biogas than sewage sludge."

The paragraphs between lines 296-312 are modified by:

Paragraph: "The way the temperature affected substrate digestion was completely opposite. The temperature increase had a negative influence on microalgae digestion; under thermophilic conditions, *I. galbana* and *S. capricornutum* reached 261±11 mL_{Biogas}/g_{VS} and 185±7 mL_{Biogas}/g_{VS} respectively, which represents a 40.5% and a 31.7% decrease compared with their biogas productions at 33 ºC. Varel et al. observed the same effect during anaerobic digestion of *Spirulina maxima*, but also process instability, decreasing the biogas production and its methane content [28]. On the contrary, some authors reported beneficial effects of the temperature increase. For example, Golueke et al. [10] reported increases in gas production and volatile matter destruction due to a temperature increase from 35 ºC to 50 ºC. El-Mashad [26] observed higher biogas production from *Spirulina platensis* when the temperature changed from 35 ºC to 50 ºC, but the methane content was lower and the methane production resulted similar. It seems that the effects of the temperature over anaerobic digestion are related to species characteristics.

As mentioned before, the temperature affected sewage sludge digestion differently and the temperature increase improved sludge digestion. Biogas production reached 566±5 mL_{Biogas}/g_{VS}, indicating that a 25.5% more biogas was produced by increasing temperature. Consequently, the way in which the temperature affects substrates digestion intensifies the differences between biogas production from microalgae and sludge; whereas *I. galbana* and *S. capricornutum* produced around 97% and 60% of the amount of biogas produced from sludge under mesophilic conditions, under thermophilic conditions these percentages were only 46% and 33%.

Answers to Associate Editor's comments.

The language of the manuscript was checked by someone who speaks English as a first language. Language corrections are marked in yellow.

Highlights

- At 33 ºC, *Isochrysis galbana* biogas yield is as high as sludge yield.
- *Selenastrum capricornutum* shows the lower biogas yield under mesophilic conditions.
- Under thermophilic condition, both microalgae species show poor digestibility.
- Biogas production is not improved by co-digestion.
- Temperature increase improves approximately by 5% the methane content in biogas.

Biogas production from sewage sludge and microalgae co-digestion under mesophilic and thermophilic conditions.

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Abstract

Isochrysis galbana and *Selenastrum capricornutum*, marine and freshwater microalgae species respectively, were co-digested with sewage sludge under mesophilic and thermophilic conditions. The substrates and the temperatures significantly influenced biogas production.

Under mesophilic conditions, the sewage sludge digestion produced 451±12 mL_{Biogas}/g_{SV}. Furthermore, all digesters were fed with *I. galbana*, or mixed with sludge, resulting in an average of 440±25 mL_{Biogas}/g_{SV}. On the contrary, *S. capricornutum*

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produced 271±6 mL_{Biogas}/g_{SV} and in the mixtures containing sludge produced intermediate values between sludge and microalgae production.

Under thermophilic conditions, the sewage sludge digestion achieved yet the highest biogas yield, 566±5 mL_{Biogas}/g_{SV}. During co-digestion, biogas production decreased when the microalgae content increased, and for *I. galbana* and for *S. capricornutum* it reached minimum values, 261±11 and 185±7 $mL_{Biogas}/g_{SV,}$ respectively. However, no evidence of inhibition was found and the low yields were attributed to microalgae species characteristics.

The methane content in biogas showed similar values, independently from the digested substrate, although this increased by approximately 5% under thermophilic condition.

Keywords:

Biogas, Biomethane potential (BMP), Co-digestion, *Isochrysis galbana*, *Selenastrum capricornutum*, Sewage sludge.

1. INTRODUCTION

The industrialisation process and the current population growth have had an immense impact on the environment. The demand on water and petroleum-based fuels are clear evidence of the increase on natural resources.

The wastewater treatment plants (WWTPs) have become an essential component in society to ensure necessary water supplies. A by product produced in these facilities during the wastewater treatment process is sewage sludge; hence as demand grows so does this by-product. The final disposal of sludge is a problem in WWTPs as this can represent up to 50% of the operating cost [1]. The anaerobic digestion of the sludge is

one of the most widespread stabilization processes in WWTPs, which converts sludge into a stable product and simultaneously recovers energy by biogas generation. On the other hand, WWTPs are a potential source of nutrients for microalgae growth: CO_2 generated and released in the atmosphere when biogas is burned; also nitrogen and phosphorus are present in wastewater [2-5].

Microalgae arose as a source of valuable chemical productions, but maybe the main feature was as promising feedstocks for renewable biofuels. In 2008, 88% of world energy demand was supplied by fossil fuels, including oil (35%), coal (29%) and natural gas (24%) [4]. Unfortunately, coal supplies depletion is predicted by 2112 and oil and gas reserves depletion by 2042, thus a rapid transition to renewable energy is needed in the near future [6]. Biodiesel production from microalgae appeared as a solution due to the microalgae advantages over the feedstocks currently used in the biodiesel industry; however, the process scale-up is unviable nowadays without a cost-effective dewatering method. Additionally, microalgae cultivation is not simple, although they grow naturally in aquatic environments. The low cell densities required for light penetration to ensure their growth and the small size of the cells are counterproductive to the harvesting step, which can represent between 20 and 30% of the total biomass production costs [4,7].

The anaerobic digestion process creates an alternative for energy recovery from microalgae. The ability to process wet biomass avoids a drying step, thus reducing large amounts of energy input. Besides, all microalgae compounds can be turned into biogas and those species unsuitable for biodiesel production due to their low oil content become potential substrates [8]. During anaerobic digestion, the organic nitrogen and phosphorus initially as biomass constituents are converted into ammonium and

phosphate, and can be recycled for microalgae cultivation reducing fertilizer needs [7]. Under these circumstances, the possibility of microalgae growth followed by anaerobic digestion in WWTPs is currently being assessed [9].

Since Golueke et al. [10] studied anaerobic digestion of microalgae for the first time, at the end of the 1950s, many microalgae species and process conditions have been evaluated for biogas production. However, not all species have the potential to produce high amounts of methane, and the proper selection of species and operating conditions are the key for biogas production [11]. The cell wall structure and composition, the carbon to nitrogen ratio (C/N), are crucial for microalgae degradability [8]. Cell wall resistance may act as a barrier hampering microorganisms being attacked; on some occasions, pre-treatment methods are required to improve digestibility [12,13]. The C/N balance, low in microalgae derived from their high protein content, increased ammonia and volatile fatty acids concentration and may potentially inhibit anaerobic digestion. Co-digestion with carbonaceous-rich waste is a method to balance this ratio and overcome this disadvantage. Sewage sludge, waste activated sludge, waste paper and corn straw are examples of increasing biogas production from different microalgae species [14-17].

In this context, this paper attempts to evaluate biogas produced from two microalgae species: the marine species *I. galbana*, and the freshwater species *S. capricornutum*, by their co-digestion with sewage sludge. Initially, the influence of the microalgae to sludge ratio and the digestion temperature over the biogas production and its methane content was evaluated.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Sewage sludge

The sludge sample consists of a primary and secondary blend, in ratio 65:35 v/v. It was collected from the municipal WWTPs in Reus (Tarragona, Spain) designed to process approximately 25,000 m^3 of wastewater daily. Sludge was received weekly and immediately stored at 4 ºC in a fridge prior to use. The experiment involved the use of two different kinds of reactors, semi-continuous reactors for acclimation and batch reactors for co-digestion; the same type of sludge was utilised in both reactors. For the first reactors the maximum storage time was a week; for the second, the maximum storage time was 2 days in order to avoid major changes on its composition or properties.

2.1.2. Microalgae

Two microalgae species were used in the experiments: *Isochrysis galbana*, marine species, and *Selenastrum capricornutum*, freshwater species. Both species were provided by the Institute for Research and Technology in Food and Agriculture IRTA (San Carles de la Ràpita, Spain). For the marine species cultivation, Walne's medium [18] was prepared with filtered (0.45 µm), autoclaved seawater from Alfacs Bay (Ebro Delta, Spain). For the freshwater species cultivation, Woods Hole MBL medium [19] was prepared with deionised water, and autoclaved before inoculation. The microalgae were cultured under batch conditions in 6 L volumetric flasks. The volumetric flasks were kept in an isothermal chamber at 20±3ºC under continuous irradiance of 120-150 µmol photons m^{-2} sec^{-1}, provided by cool-white fluorescent lamps (Philips TLD 58w/865). Mixing was provided by air flow and air was enriched by 0.7% CO_2 addition.

The microalgae were grown under these conditions for approximately 2 weeks, until the culture reached a plateau in terms of absorbance (680/800 nm).

2.1.3. Inoculum

From the start, inoculum was provided by the municipal WWTP in Reus (Tarragona, Spain). It consisted of digested sludge from mesophilic and thermophilic anaerobic reactors under continuous operating conditions. Although acclimation to new conditions was not strictly required, an anaerobic semi-continuous plant was set up to adapt inoculum to more stable temperatures, 33 ºC and 50 ºC. Four reactors (5 L each) were placed in a thermostatic bath for temperature control under magnetic stirring. Biogas production was continuously registered by volumetric gas flow meters. All reactors were daily fed with sludge, following effluent withdrawal.

Prior to the co-digestion experiments, the inoculum had previously been "degassed" by incubation at a constant temperature without feeding, to reduce the residual biodegradable organic material [20]. Observation indicated no significant methane being produced after 5 days incubation.

2.2. Experimental procedure

2.2.1. Microalgae preparation

Once microalgae were received, the biomass was collected by centrifugation using a centrifuge with a capacity of 240 mL sample (Digicen 20, Orto Alresa Centrifuges). Microalgae were recovered after 4 minutes at 10,304 RCF without temperature control. The supernatant was removed and only the pellet was recovered. Deionised water was added to recover the pellet and the total solid (TS) content of the microalgae suspension

was around 10 g/L. The suspension was stored at 4 ºC prior to use, and always used before 12 hours after microalgae centrifugation finished.

2.2.2. Co-digestion procedure

The batch reactors were set up following the procedure described by Angelidaki et al [20]. The temperature for the experiments was chosen in accordance to the temperature in the WWTP reactors, 33 ºC in the mesophilic range and 50 ºC in thermophilic range.

All experiments of co-digestion were conducted in 120 mL serum bottles in triplicate. To carry out the digestion, the microorganisms were provided with 50 mL "degassed" inoculum and optimal environmental conditions were assured by 10 mL anaerobic basic medium addition. The substrates were sewage sludge and microalgae, separately or mixed. Blank assays were prepared without substrate addition, and the biogas production was subtracted from the reactors fed with the substrates. The specific methanogenic activity for inoculum was determined with an initial concentration of 1 g/L acetic acid in the reactor.

Sludge and microalgae were added according to the experimental design. The total substrate amount was decided at 0.12 g volatile solids (VS) equivalent to 2-3 g_{COD}/L, which generates a measurable but not excessive biogas volume. The reactor feed was based on 100% sludge VS for sewage sludge digestion (denoted as Sludge in the figures), and subsequently 25%, 50%, 75% and 100% of the sludge VS were replaced with microalgae VS respectively (denoted as 25%, 50%, 75% and 100% in the figures). Deionised water was added to a final volume of 80 mL and the reactors were closed with a septum and an aluminium crimp. Finally, the reactors were purged with nitrogen to assure anaerobic conditions and placed into an oven.

Biogas production was volumetrically measured by liquid displacement. As a barrier solution, a saline solution consisting of 200 g/L NaCl and 5 g/L citric acid was used. Prior to measurements, the reactors were removed from the oven and left to reach room temperature. It was imperative that the room temperature be record in conjunction with the measurement of the biogas volume to provide results under standard conditions. Before returning the reactors to the oven, they were gently shaken. The experiment being considered finished when the biogas production was negligible.

2.3. Analytical techniques

Total solids (TS) and volatile solids (VS) were analysed according to standard methods 2540B and 2540E respectively [20]. The chemical oxygen demand (COD) was measured in a UV-spectrophotometer (DINKO UV-VIS 800 spectrophotometer) according to the standard method 5220D [21].

The protein content in the microalgae was quantified by the Lowry method [22], pre-treating samples at 100 ºC for 10 minutes with 2 N NaOH. The total sugar amount was quantified by phenol–sulfuric acid method [23]. Microalgae lipids were determined using the Bligh and Dyer method [24]. In order to assess microalgae biodegradability, the theoretical biogas potential was calculated following Buswell's equation for microalgae proteins, lipids and carbohydrates [25]. Biodegradability was expressed as measured to theoretical methane production ratio.

For the carbon to nitrogen ratio (C/N), carbon was estimated by dividing the organic matter content by 1.724 and nitrogen, dividing the protein content by 6.25 [26].

Once the experiments were concluded, volatile fatty acids (VFA) were analysed in the soluble phase by using an Agilent gas chromatograph 6890GC equipped with a flame-

ionization detector (FID). The method was performed according to Application Note 228-398 [27] from Agilent Technologies online library. An ion selective electrode (ISE) was used for ammonia concentration determination (Ammonia Gas Sensing combination electrode, mod. 51927-00, HACH).The biogas composition was analysed in a gas chromatograph (Agilent gas chromatograph 6890GC) with manual injection and a thermal conductivity detector (TCD). The separation was achieved in a porapak q 50/80 packed column 3.6m × 6.35 mm × 0.4 mm (Agilent Part No. CP99960C), using helium as carrier gas. The injector was set at a temperature of 40 ºC. The sample volume was 1 mL. The oven temperature programme started at 40 ºC and after 2 minutes was increased by 22 ºC/min to 150 ºC and held for 4 minutes. The standard used for identification and quantification of biogas components was supplied by Carburos Metálicos S.A. It consists of a mixture of methane (60% v/v), carbon dioxide (35% v/v), hydrogen (2% v/v) and hydrogen sulphide (3% v/v). Only methane and carbon dioxide were quantified, and the results were expressed as the methane percentage in a two component mixture.

The digestate was observed by Scanning Electron Microscopy (SEM) to determine the presence of entire microalgae cells which could remain after digestion. A Jeol JSM-6400 SEM was used for this purpose. A drop of each digestate sample was deposited on the support, dried at room temperature and then coated under vacuum with a gold layer before examination.

Characteristics of the sludge, the microalgae species and the inoculum used in the experiments can be seen in Table 1.

3. RESULTS AND DISCUSSION

3.1. Biogas production under mesophilic conditions

The biogas production was reported as the volume of biogas at standard conditions, 0 ºC and 1 atm, per gram VS fed, mL_{Biogas}/g_{VS}. Figure 1 shows accumulated biogas production at 33 ºC for the marine species *I. galbana*, and Figure 2, for the freshwater species *S. capricornutum*.

As it can be seen in Figure 1, the biogas production between reactors did not differ significantly, either when microalgae and sludge were digested separately or mixed. After 35 days, *I. galbana* digestion produced 439±4 mL_{Biogas}/g_{VS}. This value is higher than the reported for other saltwater microalgae like *Spirulina maxima* (330 mL_{Biogas}/g_{VS}) and for *Gracilaria* species and strains (280 and 400 mL_{Biogas}/g_{VS} respectively) [28,29]. In the same experiment, sewage sludge digestion produced 451±12 mL_{Biogas}/g_{VS}.; this value is in agreement with the values reported for sludge anaerobic digestion [1]. The comparison of microalgae and sewage sludge digestion highlights that microalgae are able to produce more or less the same quantity of biogas than sewage sludge, a substrate currently used in anaerobic digestion. In particular, *I. galbana* produces the same amount of biogas, demonstrating that this microalgae species is an eligible substrate for anaerobic digestion, competing with sewage sludge. This fact is confirmed by the results obtained during the anaerobic digestion of *Phaeodactylum tricornutum* in continuous mode (530 mL_{Biogas}/g_{VS}) and during the anaerobic digestion of *Arthrosira platensis* and *Dunaliella salina* in batch digesters (480 and 505 mL_{Biogas}/g_{VS} for respectively) [11,29]. The authors suggested that anaerobic digestion constitutes the best option to recover energy from these microalgae species.

Furthermore, when a substrate mixture was fed, biogas production showed quite similar values for all experiments, independently of the sludge to microalgae ratio in the

mixture. The averaged biogas production was 440±25 mL_{Biogas}/g_{VS}. The mixture with 25% microalgae produced the lower biogas yield, 413±7 mL_{Biogas}/g_{VS}. However, no evidence of unbalanced digestion conditions was found in these reactors. The yield drop barely represents 9% compared to the sludge or the microalgae yields, and may be attributed to experimental differences during gas volume measurements. Consequently, it was expected that by mixing the substrate the biogas yields would increase, but the results do not indicate synergistic effects. The literature relating to microalgae co-digestion showed the possibility of increasing the biogas production by mixing microalgae and sludge, due to the improved C to N ratio [10,17]. Recently, Wang et al. [14] found that *Chlorella sp.* digestion reached a considerable low biogas yield compared to waste activated sludge (WAS) digestion, but microalgae and WAS mixtures reached biogas yields similar to WAS. Hence, suggestions were put forward that the WAS addition increases the microbial activity instead of improving the C to N ratio, thus affecting microalgae cell hydrolysis. In our experiments, microalgae digestion was not improved by WAS presence in the substrate.

During the course of the experiment, it was noted that after 12 days all reactors had already exceeded 350 mL_{Biogas}/g_{VS} which represents more than 80% of the total biogas produced at the end of the experiment. Any influence of substrate mixing in the hydrolysis rate can be also dismissed.

According to Buswell's equation, conversion of proteins, lipids and carbohydrates produce 496 mL_{CH4}/g_{VS}, 1,014 mL_{CH4}/g_{VS} and 415 mL_{CH4}/g_{VS} respectively [25]. Based on the *I. galbana* composition, the theoretical methane yield is 612 mL_{CH4}/g_{VS} and the biodegradability is 55.2%. This is a high value according to conversion degrees obtained under practical conditions for highly particulate or structural substrates, and

259 can be explained by the microalgae species characteristics [25]. Additionally, it is fairly
260 well known that the microalgae cell wall determines the potential conversion into
261 biogas; *I. galbana* cells have been described as small size cells, with a lack of a cell wall
262 which facilitates anaerobic digestion [11,31]. Furthermore, the change from a high
263 saline to a non saline environment favours microalgae disintegration [30].
264 Barely 271±6 mL_{Biogas}/g_{VS} were produced during the experiments with *S. capricornutum*; this
265 production is the lowest obtained under mesophilic conditions (Figure 2). Although higher
266 biogas productions were reported for freshwater microalgae species, similar results were
267 observed in species characterised by carbohydrate-based cell walls, like in *S. capricornutum*
268 [34]. Mussgnug et al. digested different microalgae species and observed that the species
269 *Scenedesmus obliquus* and *Chlorella kessleri*, both with carbohydrate-based cell walls, showed
270 lower biogas production and higher amount of indigestible residues than species without cell
271 wall or with protein-based cell walls digested in the same conditions [11]. Even a lower biogas
272 production was reported during *Scenedesmus sp*. digestion, only 75 mL_{Biogas}/g_{VS} after 35 days at
273 mesophilic temperatures [32]. On the other side, comparison with biogas production from
274 sewage sludge, digested in the same experiment, shows that *S. capricornutum* reached around
275 60% of the biogas production from sewage sludge, 451±12 mL_{Biogas}/g_{VS}. This result differs from
276 the saltwater species *I. galbana*, which produced more or less the same quantity of biogas than
277 sewage sludge.
278 In the case of substrate mixture productions were between sludge and microalgae
279 production. Their digestion reached 394±14 mL_{Biogas}/g_{VS}, 392±4 mL_{Biogas}/g_{VS} and 330±6
280 mL_{Biogas}/g_{VS} for 25%, 50% and 75% microalgae respectively. It is noticeable that the
281 higher the amount of VS from microalgae, the lower the biogas production. Hence,
282 substrate mixing does not increase the biogas yield of the substrates separately digested
283 as was expected.

During the course of the experiment, the biogas production rate followed approximately the same tendency independently of the reactor feed composition (Figure 2). The exception being reactors fed only with microalgae, which reached 76% of the total biogas produced in the experiments after 12 days digestion, all other reactors reached 80% of the total biogas produced for the same period.

S. capricornutum has a remarkable ability to accumulate lipids with a potential for biodiesel production. This characteristic also showed a positive influence in relation to the potential methane yield, due to the high methane yield of lipids [25,33]. The theoretical biogas potential for *S. capricornutum* was estimated at 693 mL_{CH4}/g_{VS} employing Buswell's equation. Biodegradability was 30.1%, a low value compared to the marine counterpart. *S. capricornutum* cell walls are thick and rigid carbohydrate-based structures, which act as strong barriers for microorganism reducing cell degradation [34]. Additionally, the carbohydrate-based cell wall species can resist degradation better than protein-based cell wall microalgae or microalgae without a cell wall [11].

The results obtained by digesting sewage sludge and microalgae mixtures were not as expected, and the biogas yields of these mixtures shows that the process occurred without synergy between substrates.

The marine microalgae digestion performs better than the freshwater species and similar to the sludge digestion under mesophilic conditions. Unfortunately, in an integrated system for wastewater treatment, microalgae cultivation and anaerobic digestion, freshwater microalgae cultivation does not require a seawater system, making system integration easier.

3.2. Biogas production under thermophilic conditions

The biogas production under thermophilic conditions during the co-digestion of *I. galbana* is shown in Figures 3, whereas Figure 4, shows the biogas production during the co-digestion of *S. capricornutum*. The results are given as volume of biogas at standard conditions per gram VS fed, mL_{Biogas}/g_{VS}. After 20 days, biogas production from all substrates was almost negligible, and after 27 days, biogas production from blank reactors reached the highest biogas production. The experiment was considered completed and the values obtained by day 20 were considered the final biogas production yields.

The way the temperature affected substrate digestion was completely opposite. The temperature increase had a negative influence on microalgae digestion; under thermophilic conditions, *I. galbana* and *S. capricornutum* reached 261±11 mL_{Biogas}/g_{VS} and 185±7 mL_{Biogas}/g_{VS} respectively, which represents a 40.5% and a 31.7% decrease compared with their biogas productions at 33 ºC. Varel et al. observed the same effect during anaerobic digestion of *Spirulina maxima*, but also process instability, decreasing the biogas production and its methane content [28]. On the contrary, some authors reported beneficial effects of the temperature increase. For example, Golueke et al. [10] reported increases in gas production and volatile matter destruction due to a temperature increase from 35 ºC to 50 ºC. El-Mashad [26] observed higher biogas production from *Spirulina platensis* when the temperature changed from 35 ºC to 50 ºC, but the methane content was lower and the methane production resulted similar. It seems that the effects of the temperature over anaerobic digestion are related to species characteristics.

As mentioned before, the temperature affected sewage sludge digestion differently and the temperature increase improved sludge digestion. Biogas production reached 566±5

mL_{Biogas}/g_{VS}, indicating that a 25.5% more biogas was produced by increasing temperature. Consequently, the way in which the temperature affects substrates digestion intensifies the differences between biogas production from microalgae and sludge; whereas *I. galbana* and *S. capricornutum* produced around 97% and 60% of the amount of biogas produced from sludge under mesophilic conditions, under thermophilic conditions these percentages were only 46% and 33%.

In regards to the co-digestion, it can be observed that independently of the microalgae specie, experiments present similar tendencies. The higher the VS from microalgae, the lower the biogas production. Additionally, biogas production can be estimated from the measured biogas production for both substrates individually digested. The similarity of these values to the experimental values shows no advantages of mixing substrates.

In order to determine if inhibition phenomena took place during digestion, thereby explaining the lower biogas production, ammonia and VFA concentration was determined in the reactors. The protein content may represent a high percentage of the organic matter in microalgae, and their digestion release free ammonia; high free ammonia concentration has toxic effects on microorganisms. Furthermore, the temperature increase enhances the toxic effects [8]. After digestion ends, the ammonia concentration in the reactors containing microalgae was slightly higher compared with the concentration in the blank reactors. The ammonia increase was less than 50 mg/L and ammonia concentration never exceeded 550 mg/L. These concentrations are considerably lower than 1500 mg/L, the value reported as detrimental for microorganisms [1]. VFA are intermediate compounds in anaerobic digestion and their accumulation cause process instability or failure due to methanogenic inhibition. It is reported that VFA can be toxic to methanogenic microorganisms at a concentration

range of 6.7–9.0 mol/m^3 [1]. Despite VFA were not detected under mesophilic conditions, their concentration was on average 3.04±0.45 mol/m^3 under thermophilic conditions. However, this concentration is not high enough to cause methanogenic bacteria inhibition. The analysis of these results confirms that the negative influence of temperature on microalgae digestion is not related to process instability.

When compared to the mesophilic digestion, under thermophilic conditions reactors fed with microalgae needed more time to reach 80% of the total biogas, produced at the end of the experiment. Besides, a biogas increase can be observed after 8 day microalgae digestion. Sludge digestion had produced more than 80% of its total biogas production after 8 days. Before starting the experiment, anaerobic inoculum was totally acclimated to sludge, but not to microalgae. At the beginning of the experiment, microorganisms were suddenly exposed to high concentrations of microalgae and therefore an acclimation period was necessary. After this initial period, which can be seen as a lag phase, biogas production increased but after a few days it reached the final biogas yield. During co-digestion, microorganisms may degrade sewage sludge at first and later microalgae. Although the thermophilic process shows several benefits compared to the mesophilic, it is more sensitive and thus explaining the absence of lag phase under mesophilic conditions [1].

Once again, biodegradability indicates that *I. galbana* and *S. capricornutum* produced 34.9% and 21.9% of the predicted amount of biogas respectively. Similarly to digestion under mesophilic conditions, the marine species produced more biogas than the freshwater counterpart. These results may be attributed to the salinity change in the environment and the cell wall characteristics [11,30].

Finally, since a green colour was observed at the bottom of the reactor, digestate samples were examined in a Scanning Electron Microscopy (SEM) to determine microalgae incomplete digestion. In Figures 5 and 6, the presence of microalgae cells after 30 days digestion can be seen. Microalgae cells were previously photographed in their own pure culture sources; their shape and size were determined, thus facilitating microalgae identification in the digestate samples. Although digestate samples from mesophilic reactors were also observed in SEM, no cells were found. The cells indicate low degree of decomposition and presence of indigestible residues, confirming that the decrease in biodegradability at higher temperatures is due to species characteristics.

3.3. Effect of temperature digestion on biogas quality

Since biogas is mainly a methane and carbon dioxide mixture, its quality is related to the methane content among other gases. Biogas analysis showed approximately the same methane content in all reactors operating under mesophilic conditions, averaging 77±1 %CH_4, independently substrate. Also under thermophilic all reactors showed a similar composition however, the average was 82±1 %CH_4. Comparable results were also reported for *Scenedesmus obliqqus* and *Phaeodactylum tricornutum* digestion, under mesophilic and thermophilic conditions [30]. On the contrary, in *Spirulina maxima* and *Spirulina platensis* digestion, the higher the temperature, the lower its methane content [26,28]. As with biogas production yield, for the biogas composition the species characteristics determine the most suitable process conditions.

Based on biogas composition, *I. galbana* produced 338±3 mL_{CH4}/g_{VS} and 219±10 mL_{CH4}/g_{VS} at 33 ºC and 50 ºC respectively, whereas *S. capricornutum*, produced 209±5 mL_{CH4}/g_{VS} and 152±6 mL_{CH4}/g_{VS} at 33 ºC and 50 ºC respectively. The results

403 demonstrate that mesophilic anaerobic digestion represents the best alternative to digest
404 both microalgae species despite the higher methane content produced under
405 thermophilic conditions. The situation is completely the opposite for sewage sludge,
406 since thermophilic digestion increase biogas production and methane content. The
407 amount of methane was 347±9 mL_{CH4}/g_{VS} at 33 ºC compared with the 464±4 mL_{CH4}/g_{VS}
408 at 50 ºC. However, an energy balance should be done taking into account the heating
409 requirements of the thermophilic process.
410
411 **4. CONCLUSIONS**
412 Microalgae and sludge co-digestion does not improve biogas yield in comparison with
413 individual digestion of both substrates. Neither does the microalgae to sludge ratio nor
414 does the digestion temperatures improve biogas production. However, our results
415 highlight the feasibility of the marine species *I. galbana* as an eligible substrate for
416 biogas production under mesophilic conditions, since it produces a similar amount of
417 biogas to sewage sludge. On the contrary, its freshwater counterpart *S. capricornutum*
418 produces the lowest biogas amount.
419 Under thermophilic conditions, the biogas production from both microalgae species
420 decreases by 40.5% and 31.7% for *I. galbana* and *S. capricornutum* respectively,
421 contrary to biogas production from sewage sludge which improves by 25.5%. During
422 co-digestion, the higher the microalgae content in the reactors, the lower the biogas
423 production.
424 Although the methane content increases from 77±1% to 82.1% at higher temperatures,
425 the improvement does not compensate the biogas reduction caused by the higher
426 temperature, except for sludge digestion.
427

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Figure captions

Figure 1. Marine microalgae and sludge co-digestion. Batch reactors, 33 ºC, 35 days.

Figure 2. Freshwater microalgae and sludge co-digestion. Batch reactors, 33 ºC, 35 days.

Figure 3. Marine microalgae and sludge co-digestion. Batch reactors, 50 ºC, 20 days.

Figure 4. Freshwater microalgae and sludge co-digestion. Batch reactors, 50 ºC, 20 days.

Figure 5. Marine microalgae cell presence after digestion. Batch reactors, 50 ºC, 35 days.

Figure 6. Freshwater microalgae cell presence after digestion. Batch reactors, 50 ºC, 35 days.

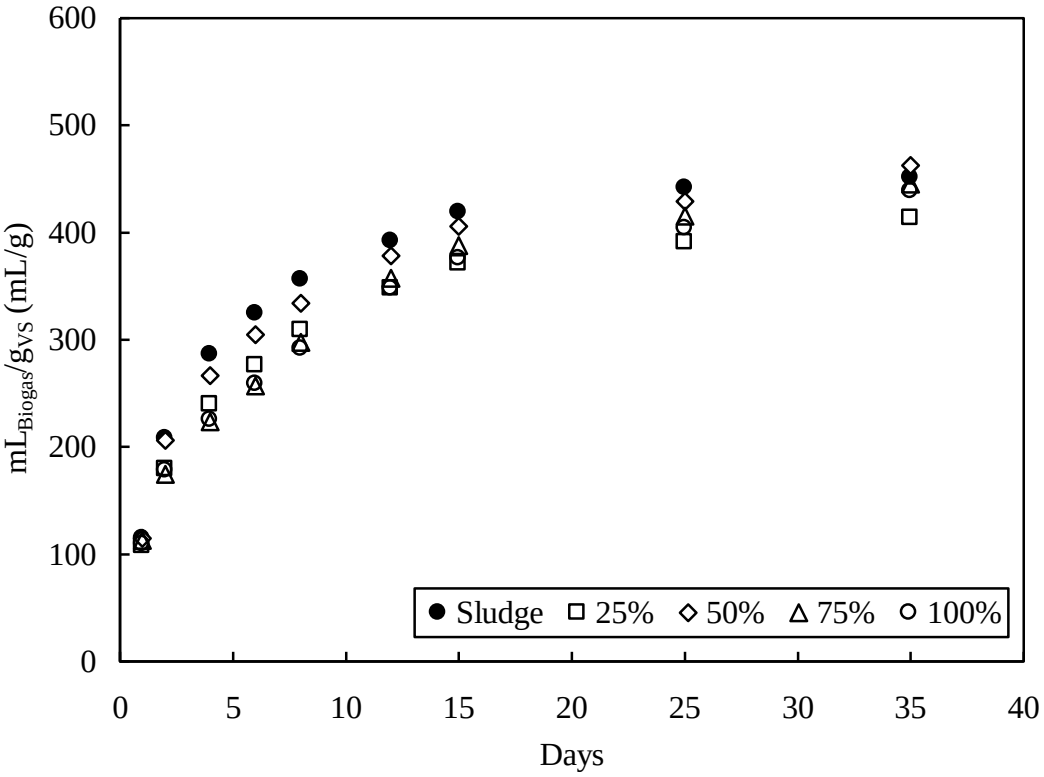
Table

Table 1. Inoculum and substrates characteristics.

Parameter	Inoculum	Sewage sludge	*Isochrysis galbana* (marine species)	*Selenastrum capricornutum* (freshwater species)
TS (g/L)	18.9±0.1	30.5±1.9	9.0-10.0[a]	9.0-10.0[a]
VS/TS	0.70	0.88	0.90	0.98
COD/VS	1.0±0.1	1.5±0.5	1.37	1.56
TN (g/100 g TS)	-	-	7.8	4.9
C:N	-	-	7.1	9.2
Proteins (g /100 g VS)	-	-	51.2	39.7
Lipids (g /100 g VS)	-	-	22.5	30.2
Carbohydrates (g /100 g VS)	-	-	15.4	29.1

[a] Microalgae were diluted with distilled water after centrifugation.

Figure 1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mL_{Biogas}/g_{VS} (mL/g)
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Days
Sludge
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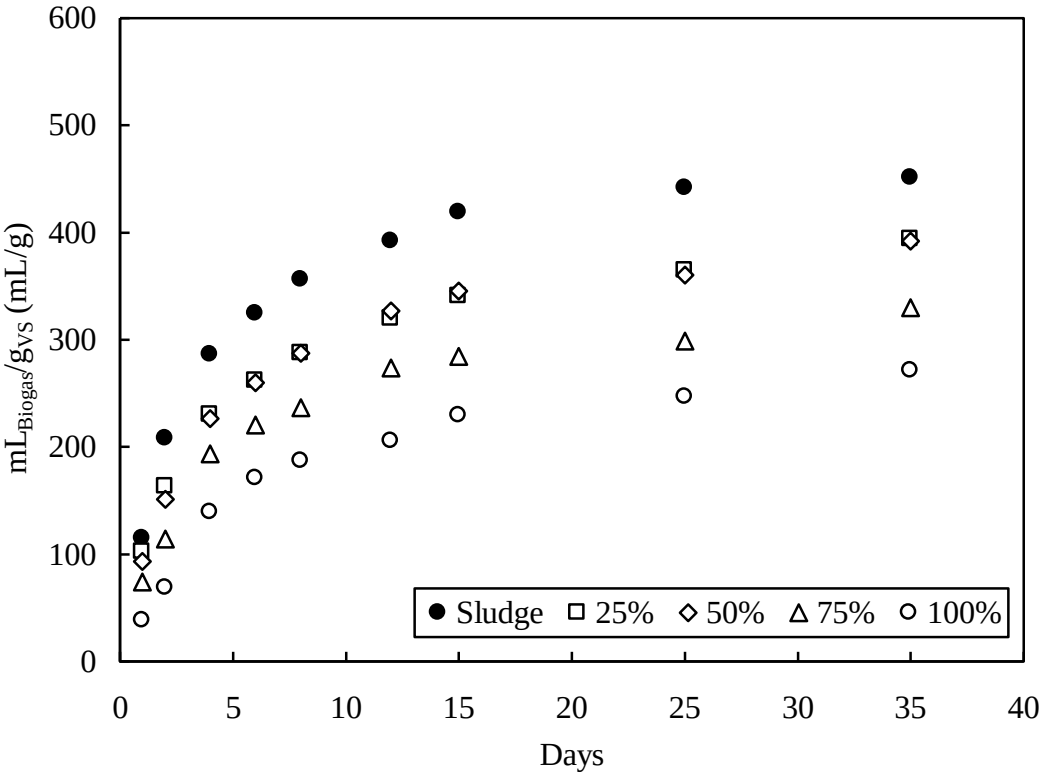
75%
100%

Figure 2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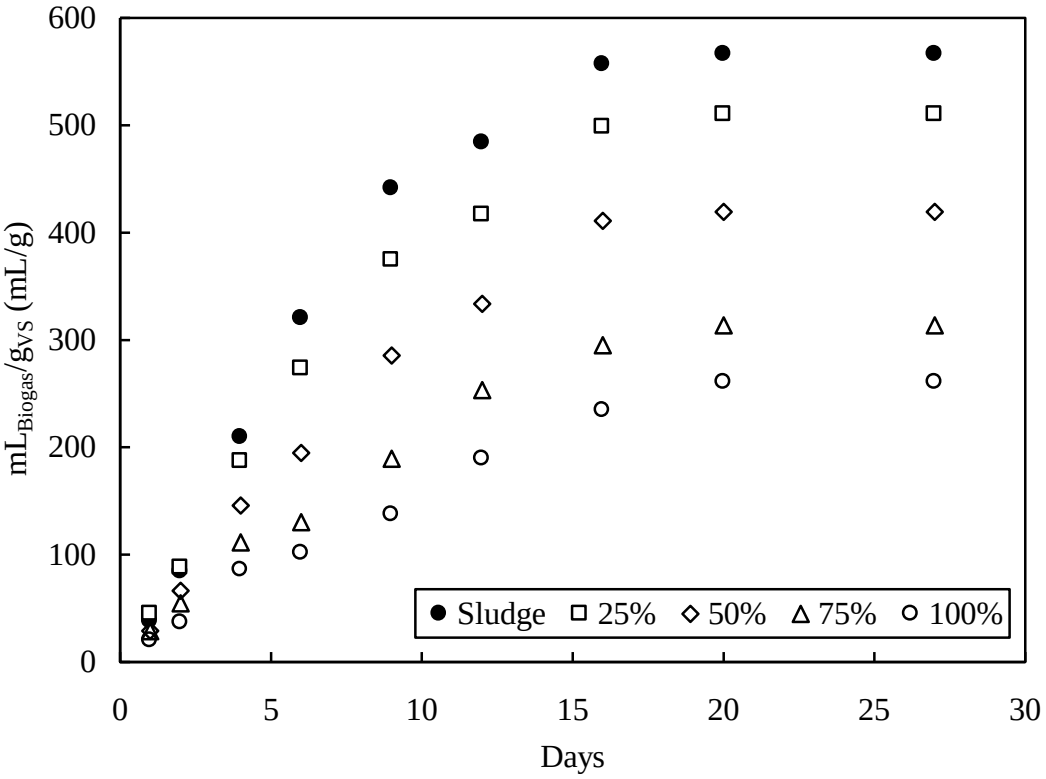
100%

Figure 3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100%

Figure 4

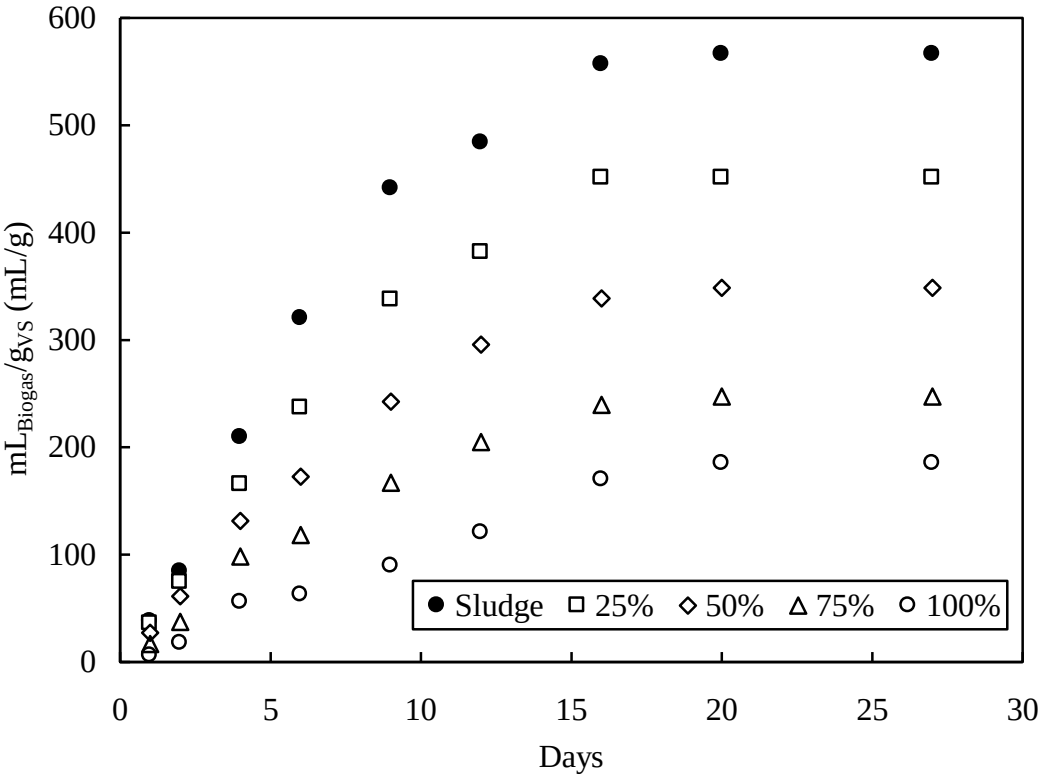
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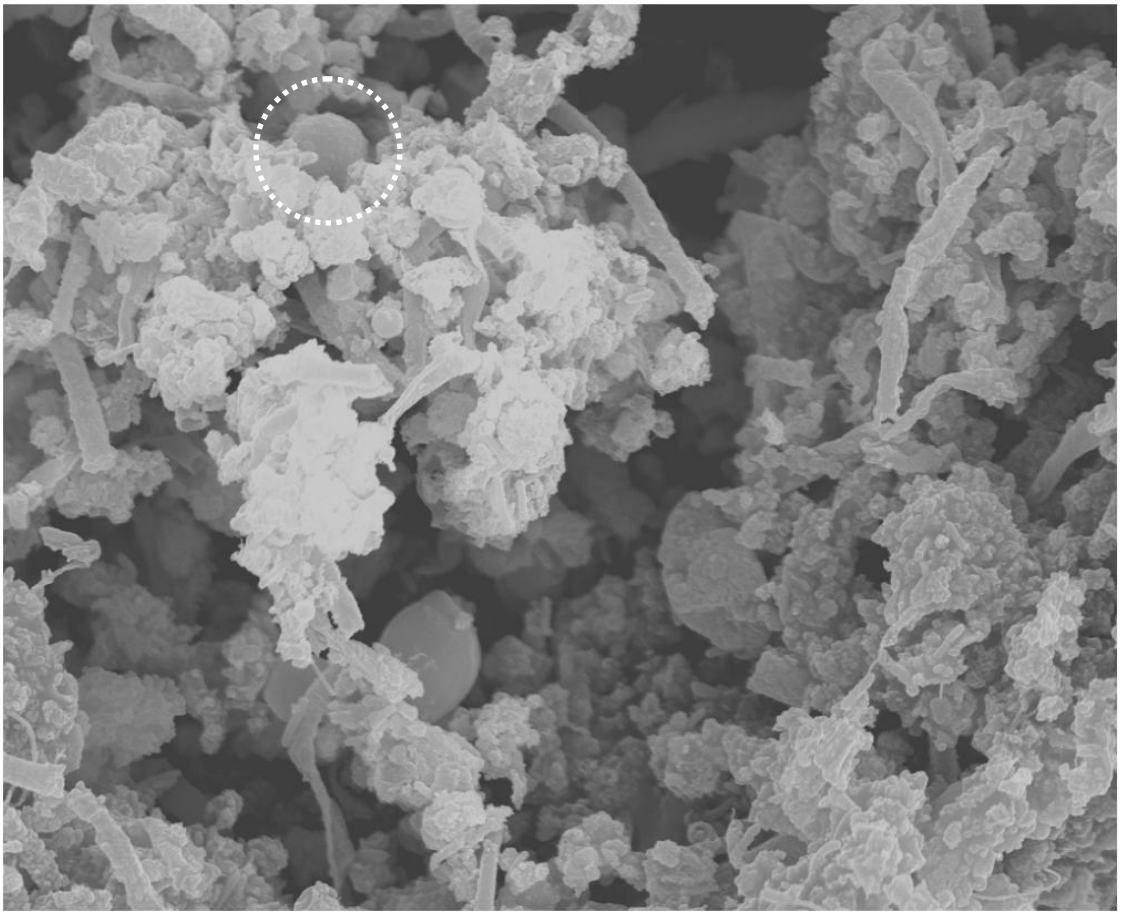
Figure 5

Figure 6



Biogas production from sewage sludge and microalgae co-digestion under mesophilic and thermophilic conditions.

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Abstract

Isochrysis galbana and *Selenastrum capricornutum*, marine and freshwater microalgae species respectively, were co-digested with sewage sludge under mesophilic and thermophilic conditions. The substrates and the temperatures significantly influenced biogas production.

Under mesophilic conditions, the sewage sludge digestion produced 451±12 mL_{Biogas}/g_{SV}. Furthermore, all digesters were fed with *I. galbana*, or mixed with sludge, resulting in an average of 440±25 mL_{Biogas}/g_{SV}. On the contrary, *S. capricornutum*

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produced 271±6 mL_{Biogas}/g_{SV} and in the mixtures containing sludge produced intermediate values between sludge and microalgae production.

Under thermophilic conditions, the sewage sludge digestion achieved yet the highest biogas yield, 566±5 mL_{Biogas}/g_{SV}. During co-digestion, biogas production decreased when the microalgae content increased, and for *I. galbana* and for *S. capricornutum* it reached minimum values, 261±11 and 185±7 $mL_{Biogas}/g_{SV,}$ respectively. However, no evidence of inhibition was found and the low yields were attributed to microalgae species characteristics.

The methane content in biogas showed similar values, independently from the digested substrate, although this increased by approximately 5% under thermophilic condition.

Keywords:

Biogas, Biomethane potential (BMP), Co-digestion, *Isochrysis galbana*, *Selenastrum capricornutum*, Sewage sludge.

1. INTRODUCTION

The industrialisation process and the current population growth have had an immense impact on the environment. The demand on water and petroleum-based fuels are clear evidence of the increase on natural resources.

The wastewater treatment plants (WWTPs) have become an essential component in society to ensure necessary water supplies. A by product produced in these facilities during the wastewater treatment process is sewage sludge; hence as demand grows so does this by-product. The final disposal of sludge is a problem in WWTPs as this can represent up to 50% of the operating cost [1]. The anaerobic digestion of the sludge is

43 one of the most widespread stabilization processes in WWTPs, which converts sludge
44 into a stable product and simultaneously recovers energy by biogas generation. On the
45 other hand, WWTPs are a potential source of nutrients for microalgae growth: CO_2
46 generated and released in the atmosphere when biogas is burned; also nitrogen and
47 phosphorus are present in wastewater [2-5].
48 Microalgae arose as a source of valuable chemical productions, but maybe the main
49 feature was as promising feedstocks for renewable biofuels. In 2008, 88% of world
50 energy demand was supplied by fossil fuels, including oil (35%), coal (29%) and natural
51 gas (24%) [4]. Unfortunately, coal supplies depletion is predicted by 2112 and oil and
52 gas reserves depletion by 2042, thus a rapid transition to renewable energy is needed in
53 the near future [6]. Biodiesel production from microalgae appeared as a solution due to
54 the microalgae advantages over the feedstocks currently used in the biodiesel industry;
55 however, the process scale-up is unviable nowadays without a cost-effective dewatering
56 method. Additionally, microalgae cultivation is not simple, although they grow
57 naturally in aquatic environments. The low cell densities required for light penetration
58 to ensure their growth and the small size of the cells are counterproductive to the
59 harvesting step, which can represent between 20 and 30% of the total biomass
60 production costs [4,7].
61 The anaerobic digestion process creates an alternative for energy recovery from
62 microalgae. The ability to process wet biomass avoids a drying step, thus reducing large
63 amounts of energy input. Besides, all microalgae compounds can be turned into biogas
64 and those species unsuitable for biodiesel production due to their low oil content
65 become potential substrates [8]. During anaerobic digestion, the organic nitrogen and
66 phosphorus initially as biomass constituents are converted into ammonium and

phosphate, and can be recycled for microalgae cultivation reducing fertilizer needs [7]. Under these circumstances, the possibility of microalgae growth followed by anaerobic digestion in WWTPs is currently being assessed [9].

Since Golueke et al. [10] studied anaerobic digestion of microalgae for the first time, at the end of the 1950s, many microalgae species and process conditions have been evaluated for biogas production. However, not all species have the potential to produce high amounts of methane, and the proper selection of species and operating conditions are the key for biogas production [11]. The cell wall structure and composition, the carbon to nitrogen ratio (C/N), are crucial for microalgae degradability [8]. Cell wall resistance may act as a barrier hampering microorganisms being attacked; on some occasions, pre-treatment methods are required to improve digestibility [12,13]. The C/N balance, low in microalgae derived from their high protein content, increased ammonia and volatile fatty acids concentration and may potentially inhibit anaerobic digestion. Co-digestion with carbonaceous-rich waste is a method to balance this ratio and overcome this disadvantage. Sewage sludge, waste activated sludge, waste paper and corn straw are examples of increasing biogas production from different microalgae species [14-17].

In this context, this paper attempts to evaluate biogas produced from two microalgae species: the marine species *I. galbana*, and the freshwater species *S. capricornutum*, by their co-digestion with sewage sludge. Initially, the influence of the microalgae to sludge ratio and the digestion temperature over the biogas production and its methane content was evaluated.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Sewage sludge

The sludge sample consists of a primary and secondary blend, in ratio 65:35 v/v. It was collected from the municipal WWTPs in Reus (Tarragona, Spain) designed to process approximately 25,000 m^3 of wastewater daily. Sludge was received weekly and immediately stored at 4 ºC in a fridge prior to use. The experiment involved the use of two different kinds of reactors, semi-continuous reactors for acclimation and batch reactors for co-digestion; the same type of sludge was utilised in both reactors. For the first reactors the maximum storage time was a week; for the second, the maximum storage time was 2 days in order to avoid major changes on its composition or properties.

2.1.2. Microalgae

Two microalgae species were used in the experiments: *Isochrysis galbana*, marine species, and *Selenastrum capricornutum*, freshwater species. Both species were provided by the Institute for Research and Technology in Food and Agriculture IRTA (San Carles de la Ràpita, Spain). For the marine species cultivation, Walne's medium [18] was prepared with filtered (0.45 µm), autoclaved seawater from Alfacs Bay (Ebro Delta, Spain). For the freshwater species cultivation, Woods Hole MBL medium [19] was prepared with deionised water, and autoclaved before inoculation. The microalgae were cultured under batch conditions in 6 L volumetric flasks. The volumetric flasks were kept in an isothermal chamber at 20±3ºC under continuous irradiance of 120-150 µmol photons m^{-2} sec^{-1}, provided by cool-white fluorescent lamps (Philips TLD 58w/865). Mixing was provided by air flow and air was enriched by 0.7% CO_2 addition.

The microalgae were grown under these conditions for approximately 2 weeks, until the culture reached a plateau in terms of absorbance (680/800 nm).

2.1.3. Inoculum

From the start, inoculum was provided by the municipal WWTP in Reus (Tarragona, Spain). It consisted of digested sludge from mesophilic and thermophilic anaerobic reactors under continuous operating conditions. Although acclimation to new conditions was not strictly required, an anaerobic semi-continuous plant was set up to adapt inoculum to more stable temperatures, 33 ºC and 50 ºC. Four reactors (5 L each) were placed in a thermostatic bath for temperature control under magnetic stirring. Biogas production was continuously registered by volumetric gas flow meters. All reactors were daily fed with sludge, following effluent withdrawal.

Prior to the co-digestion experiments, the inoculum had previously been "degassed" by incubation at a constant temperature without feeding, to reduce the residual biodegradable organic material [20]. Observation indicated no significant methane being produced after 5 days incubation.

2.2. Experimental procedure

2.2.1. Microalgae preparation

Once microalgae were received, the biomass was collected by centrifugation using a centrifuge with a capacity of 240 mL sample (Digicen 20, Orto Alresa Centrifuges). Microalgae were recovered after 4 minutes at 10,304 RCF without temperature control. The supernatant was removed and only the pellet was recovered. Deionised water was added to recover the pellet and the total solid (TS) content of the microalgae suspension

was around 10 g/L. The suspension was stored at 4 ºC prior to use, and always used before 12 hours after microalgae centrifugation finished.

2.2.2. Co-digestion procedure

The batch reactors were set up following the procedure described by Angelidaki et al [20]. The temperature for the experiments was chosen in accordance to the temperature in the WWTP reactors, 33 ºC in the mesophilic range and 50 ºC in thermophilic range.

All experiments of co-digestion were conducted in 120 mL serum bottles in triplicate. To carry out the digestion, the microorganisms were provided with 50 mL "degassed" inoculum and optimal environmental conditions were assured by 10 mL anaerobic basic medium addition. The substrates were sewage sludge and microalgae, separately or mixed. Blank assays were prepared without substrate addition, and the biogas production was subtracted from the reactors fed with the substrates. The specific methanogenic activity for inoculum was determined with an initial concentration of 1 g/L acetic acid in the reactor.

Sludge and microalgae were added according to the experimental design. The total substrate amount was decided at 0.12 g volatile solids (VS) equivalent to 2-3 g_{COD}/L, which generates a measurable but not excessive biogas volume. The reactor feed was based on 100% sludge VS for sewage sludge digestion (denoted as Sludge in the figures), and subsequently 25%, 50%, 75% and 100% of the sludge VS were replaced with microalgae VS respectively (denoted as 25%, 50%, 75% and 100% in the figures). Deionised water was added to a final volume of 80 mL and the reactors were closed with a septum and an aluminium crimp. Finally, the reactors were purged with nitrogen to assure anaerobic conditions and placed into an oven.

Biogas production was volumetrically measured by liquid displacement. As a barrier solution, a saline solution consisting of 200 g/L NaCl and 5 g/L citric acid was used. Prior to measurements, the reactors were removed from the oven and left to reach room temperature. It was imperative that the room temperature be record in conjunction with the measurement of the biogas volume to provide results under standard conditions. Before returning the reactors to the oven, they were gently shaken. The experiment being considered finished when the biogas production was negligible.

2.3. Analytical techniques

Total solids (TS) and volatile solids (VS) were analysed according to standard methods 2540B and 2540E respectively [20]. The chemical oxygen demand (COD) was measured in a UV-spectrophotometer (DINKO UV-VIS 800 spectrophotometer) according to the standard method 5220D [21].

The protein content in the microalgae was quantified by the Lowry method [22], pre-treating samples at 100 ºC for 10 minutes with 2 N NaOH. The total sugar amount was quantified by phenol–sulfuric acid method [23]. Microalgae lipids were determined using the Bligh and Dyer method [24]. In order to assess microalgae biodegradability, the theoretical biogas potential was calculated following Buswell's equation for microalgae proteins, lipids and carbohydrates [25]. Biodegradability was expressed as measured to theoretical methane production ratio.

For the carbon to nitrogen ratio (C/N), carbon was estimated by dividing the organic matter content by 1.724 and nitrogen, dividing the protein content by 6.25 [26].

Once the experiments were concluded, volatile fatty acids (VFA) were analysed in the soluble phase by using an Agilent gas chromatograph 6890GC equipped with a flame-

ionization detector (FID). The method was performed according to Application Note 228-398 [27] from Agilent Technologies online library. An ion selective electrode (ISE) was used for ammonia concentration determination (Ammonia Gas Sensing combination electrode, mod. 51927-00, HACH).The biogas composition was analysed in a gas chromatograph (Agilent gas chromatograph 6890GC) with manual injection and a thermal conductivity detector (TCD). The separation was achieved in a porapak q 50/80 packed column 3.6m × 6.35 mm × 0.4 mm (Agilent Part No. CP99960C), using helium as carrier gas. The injector was set at a temperature of 40 ºC. The sample volume was 1 mL. The oven temperature programme started at 40 ºC and after 2 minutes was increased by 22 ºC/min to 150 ºC and held for 4 minutes. The standard used for identification and quantification of biogas components was supplied by Carburos Metálicos S.A. It consists of a mixture of methane (60% v/v), carbon dioxide (35% v/v), hydrogen (2% v/v) and hydrogen sulphide (3% v/v). Only methane and carbon dioxide were quantified, and the results were expressed as the methane percentage in a two component mixture.

The digestate was observed by Scanning Electron Microscopy (SEM) to determine the presence of entire microalgae cells which could remain after digestion. A Jeol JSM-6400 SEM was used for this purpose. A drop of each digestate sample was deposited on the support, dried at room temperature and then coated under vacuum with a gold layer before examination.

Characteristics of the sludge, the microalgae species and the inoculum used in the experiments can be seen in Table 1.

3. RESULTS AND DISCUSSION

3.1. Biogas production under mesophilic conditions

The biogas production was reported as the volume of biogas at standard conditions, 0 ºC and 1 atm, per gram VS fed, mL_{Biogas}/g_{VS}. Figure 1 shows accumulated biogas production at 33 ºC for the marine species *I. galbana*, and Figure 2, for the freshwater species *S. capricornutum*.

As it can be seen in Figure 1, the biogas production between reactors did not differ significantly, either when microalgae and sludge were digested separately or mixed. After 35 days, *I. galbana* digestion produced 439±4 mL_{Biogas}/g_{VS}. This value is higher than the reported for other saltwater microalgae like *Spirulina maxima* (330 mL_{Biogas}/g_{VS}) and for *Gracilaria* species and strains (280 and 400 mL_{Biogas}/g_{VS} respectively) [28,29]. In the same experiment, sewage sludge digestion produced 451±12 mL_{Biogas}/g_{VS}.; this value is in agreement with the values reported for sludge anaerobic digestion [1]. The comparison of microalgae and sewage sludge digestion highlights that microalgae are able to produce more or less the same quantity of biogas than sewage sludge, a substrate currently used in anaerobic digestion. In particular, *I. galbana* produces the same amount of biogas, demonstrating that this microalgae species is an eligible substrate for anaerobic digestion, competing with sewage sludge. This fact is confirmed by the results obtained during the anaerobic digestion of *Phaeodactylum tricornutum* in continuous mode (530 mL_{Biogas}/g_{VS}) and during the anaerobic digestion of *Arthrosira platensis* and *Dunaliella salina* in batch digesters (480 and 505 mL_{Biogas}/g_{VS} for respectively) [11,29]. The authors suggested that anaerobic digestion constitutes the best option to recover energy from these microalgae species.

Furthermore, when a substrate mixture was fed, biogas production showed quite similar values for all experiments, independently of the sludge to microalgae ratio in the

mixture. The averaged biogas production was 440±25 mL_{Biogas}/g_{VS}. The mixture with 25% microalgae produced the lower biogas yield, 413±7 mL_{Biogas}/g_{VS}. However, no evidence of unbalanced digestion conditions was found in these reactors. The yield drop barely represents 9% compared to the sludge or the microalgae yields, and may be attributed to experimental differences during gas volume measurements. Consequently, it was expected that by mixing the substrate the biogas yields would increase, but the results do not indicate synergistic effects. The literature relating to microalgae co-digestion showed the possibility of increasing the biogas production by mixing microalgae and sludge, due to the improved C to N ratio [10,17]. Recently, Wang et al. [14] found that *Chlorella sp.* digestion reached a considerable low biogas yield compared to waste activated sludge (WAS) digestion, but microalgae and WAS mixtures reached biogas yields similar to WAS. Hence, suggestions were put forward that the WAS addition increases the microbial activity instead of improving the C to N ratio, thus affecting microalgae cell hydrolysis. In our experiments, microalgae digestion was not improved by WAS presence in the substrate.

During the course of the experiment, it was noted that after 12 days all reactors had already exceeded 350 mL_{Biogas}/g_{VS} which represents more than 80% of the total biogas produced at the end of the experiment. Any influence of substrate mixing in the hydrolysis rate can be also dismissed.

According to Buswell's equation, conversion of proteins, lipids and carbohydrates produce 496 mL_{CH4}/g_{VS}, 1,014 mL_{CH4}/g_{VS} and 415 mL_{CH4}/g_{VS} respectively [25]. Based on the *I. galbana* composition, the theoretical methane yield is 612 mL_{CH4}/g_{VS} and the biodegradability is 55.2%. This is a high value according to conversion degrees obtained under practical conditions for highly particulate or structural substrates, and

259 can be explained by the microalgae species characteristics [25]. Additionally, it is fairly
260 well known that the microalgae cell wall determines the potential conversion into
261 biogas; *I. galbana* cells have been described as small size cells, with a lack of a cell wall
262 which facilitates anaerobic digestion [11,31]. Furthermore, the change from a high
263 saline to a non saline environment favours microalgae disintegration [30].

264 Barely 271±6 mL_{Biogas}/g_{VS} were produced during the experiments with *S. capricornutum*; this
265 production is the lowest obtained under mesophilic conditions (Figure 2). Although higher
266 biogas productions were reported for freshwater microalgae species, similar results were
267 observed in species characterised by carbohydrate-based cell walls, like in *S. capricornutum*
268 [34]. Mussgnug et al. digested different microalgae species and observed that the species
269 *Scenedesmus obliquus* and *Chlorella kessleri*, both with carbohydrate-based cell walls, showed
270 lower biogas production and higher amount of indigestible residues than species without cell
271 wall or with protein-based cell walls digested in the same conditions [11]. Even a lower biogas
272 production was reported during *Scenedesmus sp*. digestion, only 75 mL_{Biogas}/g_{VS} after 35 days at
273 mesophilic temperatures [32]. On the other side, comparison with biogas production from
274 sewage sludge, digested in the same experiment, shows that *S. capricornutum* reached around
275 60% of the biogas production from sewage sludge, 451±12 $mL_{Biogas}/g_{VS.}$. This result differs from
276 the saltwater species *I. galbana*, which produced more or less the same quantity of biogas than
277 sewage sludge.

278 In the case of substrate mixture productions were between sludge and microalgae
279 production. Their digestion reached 394±14 mL_{Biogas}/g_{VS}, 392±4 mL_{Biogas}/g_{VS} and 330±6
280 mL_{Biogas}/g_{VS} for 25%, 50% and 75% microalgae respectively. It is noticeable that the
281 higher the amount of VS from microalgae, the lower the biogas production. Hence,
282 substrate mixing does not increase the biogas yield of the substrates separately digested
283 as was expected.

During the course of the experiment, the biogas production rate followed approximately the same tendency independently of the reactor feed composition (Figure 2). The exception being reactors fed only with microalgae, which reached 76% of the total biogas produced in the experiments after 12 days digestion, all other reactors reached 80% of the total biogas produced for the same period.

S. capricornutum has a remarkable ability to accumulate lipids with a potential for biodiesel production. This characteristic also showed a positive influence in relation to the potential methane yield, due to the high methane yield of lipids [25,33]. The theoretical biogas potential for *S. capricornutum* was estimated at 693 mL_{CH4}/g_{VS} employing Buswell's equation. Biodegradability was 30.1%, a low value compared to the marine counterpart. *S. capricornutum* cell walls are thick and rigid carbohydrate-based structures, which act as strong barriers for microorganism reducing cell degradation [34]. Additionally, the carbohydrate-based cell wall species can resist degradation better than protein-based cell wall microalgae or microalgae without a cell wall [11].

The results obtained by digesting sewage sludge and microalgae mixtures were not as expected, and the biogas yields of these mixtures shows that the process occurred without synergy between substrates.

The marine microalgae digestion performs better than the freshwater species and similar to the sludge digestion under mesophilic conditions. Unfortunately, in an integrated system for wastewater treatment, microalgae cultivation and anaerobic digestion, freshwater microalgae cultivation does not require a seawater system, making system integration easier.

3.2. Biogas production under thermophilic conditions

The biogas production under thermophilic conditions during the co-digestion of *I. galbana* is shown in Figures 3, whereas Figure 4, shows the biogas production during the co-digestion of *S. capricornutum*. The results are given as volume of biogas at standard conditions per gram VS fed, mL_{Biogas}/g_{VS}. After 20 days, biogas production from all substrates was almost negligible, and after 27 days, biogas production from blank reactors reached the highest biogas production. The experiment was considered completed and the values obtained by day 20 were considered the final biogas production yields.

The way the temperature affected substrate digestion was completely opposite. The temperature increase had a negative influence on microalgae digestion; under thermophilic conditions, *I. galbana* and *S. capricornutum* reached 261±11 mL_{Biogas}/g_{VS} and 185±7 mL_{Biogas}/g_{VS} respectively, which represents a 40.5% and a 31.7% decrease compared with their biogas productions at 33 ºC. Varel et al. observed the same effect during anaerobic digestion of *Spirulina maxima*, but also process instability, decreasing the biogas production and its methane content [28]. On the contrary, some authors reported beneficial effects of the temperature increase. For example, Golueke et al. [10] reported increases in gas production and volatile matter destruction due to a temperature increase from 35 ºC to 50 ºC. El-Mashad [26] observed higher biogas production from *Spirulina platensis* when the temperature changed from 35 ºC to 50 ºC, but the methane content was lower and the methane production resulted similar. It seems that the effects of the temperature over anaerobic digestion are related to species characteristics.

As mentioned before, the temperature affected sewage sludge digestion differently and the temperature increase improved sludge digestion. Biogas production reached 566±5

332 mL_{Biogas}/g_{VS}, indicating that a 25.5% more biogas was produced by increasing
333 temperature. Consequently, the way in which the temperature affects substrates
334 digestion intensifies the differences between biogas production from microalgae and
335 sludge; whereas *I. galbana* and *S. capricornutum* produced around 97% and 60% of the
336 amount of biogas produced from sludge under mesophilic conditions, under
337 thermophilic conditions these percentages were only 46% and 33%.
338 In regards to the co-digestion, it can be observed that independently of the microalgae
339 specie, experiments present similar tendencies. The higher the VS from microalgae, the
340 lower the biogas production. Additionally, biogas production can be estimated from the
341 measured biogas production for both substrates individually digested. The similarity of
342 these values to the experimental values shows no advantages of mixing substrates.
343 In order to determine if inhibition phenomena took place during digestion, thereby
344 explaining the lower biogas production, ammonia and VFA concentration was
345 determined in the reactors. The protein content may represent a high percentage of the
346 organic matter in microalgae, and their digestion release free ammonia; high free
347 ammonia concentration has toxic effects on microorganisms. Furthermore, the
348 temperature increase enhances the toxic effects [8]. After digestion ends, the ammonia
349 concentration in the reactors containing microalgae was slightly higher compared with
350 the concentration in the blank reactors. The ammonia increase was less than 50 mg/L
351 and ammonia concentration never exceeded 550 mg/L. These concentrations are
352 considerably lower than 1500 mg/L, the value reported as detrimental for
353 microorganisms [1]. VFA are intermediate compounds in anaerobic digestion and
354 their accumulation cause process instability or failure due to methanogenic inhibition. It
355 is reported that VFA can be toxic to methanogenic microorganisms at a concentration

range of 6.7–9.0 mol/m^3 [1]. Despite VFA were not detected under mesophilic conditions, their concentration was on average 3.04±0.45 mol/m^3 under thermophilic conditions. However, this concentration is not high enough to cause methanogenic bacteria inhibition. The analysis of these results confirms that the negative influence of temperature on microalgae digestion is not related to process instability.

When compared to the mesophilic digestion, under thermophilic conditions reactors fed with microalgae needed more time to reach 80% of the total biogas, produced at the end of the experiment. Besides, a biogas increase can be observed after 8 day microalgae digestion. Sludge digestion had produced more than 80% of its total biogas production after 8 days. Before starting the experiment, anaerobic inoculum was totally acclimated to sludge, but not to microalgae. At the beginning of the experiment, microorganisms were suddenly exposed to high concentrations of microalgae and therefore an acclimation period was necessary. After this initial period, which can be seen as a lag phase, biogas production increased but after a few days it reached the final biogas yield. During co-digestion, microorganisms may degrade sewage sludge at first and later microalgae. Although the thermophilic process shows several benefits compared to the mesophilic, it is more sensitive and thus explaining the absence of lag phase under mesophilic conditions [1].

Once again, biodegradability indicates that *I. galbana* and *S. capricornutum* produced 34.9% and 21.9% of the predicted amount of biogas respectively. Similarly to digestion under mesophilic conditions, the marine species produced more biogas than the freshwater counterpart. These results may be attributed to the salinity change in the environment and the cell wall characteristics [11,30].

Finally, since a green colour was observed at the bottom of the reactor, digestate samples were examined in a Scanning Electron Microscopy (SEM) to determine microalgae incomplete digestion. In Figures 5 and 6, the presence of microalgae cells after 30 days digestion can be seen. Microalgae cells were previously photographed in their own pure culture sources; their shape and size were determined, thus facilitating microalgae identification in the digestate samples. Although digestate samples from mesophilic reactors were also observed in SEM, no cells were found. The cells indicate low degree of decomposition and presence of indigestible residues, confirming that the decrease in biodegradability at higher temperatures is due to species characteristics.

3.3. Effect of temperature digestion on biogas quality

Since biogas is mainly a methane and carbon dioxide mixture, its quality is related to the methane content among other gases. Biogas analysis showed approximately the same methane content in all reactors operating under mesophilic conditions, averaging 77±1 $\%CH_4$, independently substrate. Also under thermophilic all reactors showed a similar composition however, the average was 82±1 $\%CH_4$. Comparable results were also reported for *Scenedesmus obliqqus* and *Phaeodactylum tricornutum* digestion, under mesophilic and thermophilic conditions [30]. On the contrary, in *Spirulina maxima* and *Spirulina platensis* digestion, the higher the temperature, the lower its methane content [26,28]. As with biogas production yield, for the biogas composition the species characteristics determine the most suitable process conditions.

Based on biogas composition, *I. galbana* produced 338±3 mL_{CH4}/g_{VS} and 219±10 mL_{CH4}/g_{VS} at 33 ºC and 50 ºC respectively, whereas *S. capricornutum*, produced 209±5 mL_{CH4}/g_{VS} and 152±6 mL_{CH4}/g_{VS} at 33 ºC and 50 ºC respectively. The results

403 demonstrate that mesophilic anaerobic digestion represents the best alternative to digest
404 both microalgae species despite the higher methane content produced under
405 thermophilic conditions. The situation is completely the opposite for sewage sludge,
406 since thermophilic digestion increase biogas production and methane content. The
407 amount of methane was 347±9 mL_{CH4}/g_{VS} at 33 ºC compared with the 464±4 mL_{CH4}/g_{VS}
408 at 50 ºC. However, an energy balance should be done taking into account the heating
409 requirements of the thermophilic process.
410

411 **4. CONCLUSIONS**

412 Microalgae and sludge co-digestion does not improve biogas yield in comparison with
413 individual digestion of both substrates. Neither does the microalgae to sludge ratio nor
414 does the digestion temperatures improve biogas production. However, our results
415 highlight the feasibility of the marine species *I. galbana* as an eligible substrate for
416 biogas production under mesophilic conditions, since it produces a similar amount of
417 biogas to sewage sludge. On the contrary, its freshwater counterpart *S. capricornutum*
418 produces the lowest biogas amount.
419 Under thermophilic conditions, the biogas production from both microalgae species
420 decreases by 40.5% and 31.7% for *I. galbana* and *S. capricornutum* respectively,
421 contrary to biogas production from sewage sludge which improves by 25.5%. During
422 co-digestion, the higher the microalgae content in the reactors, the lower the biogas
423 production.
424 Although the methane content increases from 77±1% to 82.1% at higher temperatures,
425 the improvement does not compensate the biogas reduction caused by the higher
426 temperature, except for sludge digestion.

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Figure captions

Figure 1. Marine microalgae and sludge co-digestion. Batch reactors, 33 ºC, 35 days.

Figure 2. Freshwater microalgae and sludge co-digestion. Batch reactors, 33 ºC, 35 days.

Figure 3. Marine microalgae and sludge co-digestion. Batch reactors, 50 ºC, 20 days.

Figure 4. Freshwater microalgae and sludge co-digestion. Batch reactors, 50 ºC, 20 days.

Figure 5. Marine microalgae cell presence after digestion. Batch reactors, 50 ºC, 35 days.

Figure 6. Freshwater microalgae cell presence after digestion. Batch reactors, 50 ºC, 35 days.