Anaerobic co-digestion of microalgal biomass and wheat straw with and without thermo-alkaline pretreatment

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Abstract

This study aimed at analyzing the anaerobic co-digestion of microalgal biomass grown in wastewater and wheat straw. To this end, Biochemical Methane Potential (BMP) tests were carried out testing different substrate proportions (20-80, 50-50 and 80-20%, on a volatile solid basis). In order to improve their biodegradability, the co-digestion of both substrates was also evaluated after applying a thermo-alkaline pretreatment (10% CaO at 75^{o}C for 24h). The highest synergies in degradation rates were observed by adding at least 50% of wheat straw. Therefore, the co-digestion of 50% microalgae - 50% wheat straw was investigated in mesophilic lab-scale reactors. The results showed that the methane yield was increased by 77% with the co-digestion as compared to microalgae mono-digestion, while the pretreatment only increased the methane yield by 15% compared to the untreated mixture. Thus, the anaerobic co-digestion of microalgae and wheat straw was successful even without applying a thermo-alkaline pretreatment.

Keywords: Biogas, C/N ratio; microalgae, lignocellulosic biomass, thermo-chemical pretreatment

1. Introduction

In order to overcome the world's major challenges of freshwater shortage and energy crisis, carbon- and energy-neutral wastewater treatment processes are urgently needed. Towards this goal, algae-based wastewater treatment plants (WWTPs) offer many advantages over

45 the conventional WWTPs with activated sludge process for carbon (C) and biological
46 nutrient removal (BNR) processes for nitrogen (N) and phosphorus (P) treatment.
47 Microalgae are capable of using inorganic N, P in the wastewater along with CO_2 and
48 produce biomass and oxygen through photosynthesis in the presence of sunlight. The
49 oxygen produced by microalgae can be utilized by heterotrophic bacteria within the flocs
50 for organic C removal which reduces the energy requirement of wastewater treatment and
51 provides CO_2 for microalgae (Rawat et al., 2011). Furthermore, excess algal biomass from
52 the wastewater treatment process can be digested/co-digested in anaerobic digesters
53 (Golueke et al., 1957; Ward et al., 2014) for organic matter reduction and methane-rich
54 biogas recovery prior to land application as soil amendment (Solé-Bundó et al., 2017).

55 Despite the aforementioned advantages, there are barriers to accomplish sustainable, large-
56 scale, algae-based WWTPs incorporating anaerobic digestion. First of all, volatile solids
57 (VS) removal of microalgal biomass grown in wastewater is limited to 21–36% in
58 continuously-fed anaerobic digesters at a hydraulic retention time (HRT) range of 15–20
59 days with specific methane yields of 0.10–0.18 L/ g VS (Passos and Ferrer, 2014). The low
60 conversion yield to methane is attributed to the nature of the cell structure in microalgae,
61 which is mostly composed of organic compounds with low biodegradability that creates
62 resistance to hydrolysis during anaerobic digestion. Furthermore, as the type of
63 predominant species in microalgal biomass and their growth rates are quite seasonal
64 depending on wastewater characteristics and availability of sunlight, the amount,
65 characteristics and biodegradability of algal biomass are changing throughout the year
66 (Passos et al., 2015b).

In the last 10 years, many pretreatment technologies have been investigated to break apart the complex structure of microalgae and make organics within the cell walls bioavailable to acid/methane formers to increase methane yields. A review by Passos et al. (2014) revealed that thermal (< 100^{o}C, atmospheric pressure), hydrothermal (>100^{o}C, gradual pressure release), and steam explosion (>100^{o}C, sudden pressure release) pretreatments of different microalgae species (some grown in wastewater) resulted in a wide range of improvements in methane yields (-13 to 220%). In general, pretreatments achieving high temperature (110 – 170^{o}C) and pressure (1 - 6.4 bar) via steam injection/explosion or hydrothermal ways achieved superior solubilization/methane yield results (Alzate et al., 2012). However, energy assessments rarely pointed out a feasible full-scale application unless microalgal biomass was concentrated (i.e. > 8% TS) prior to pretreatment (Passos and Ferrer, 2015). Mechanical pretreatments (i.e. ultrasound, microwave, high-pressure homogenization) were found less microalgae strain-dependent but required high energy input (i.e. 132 – 529 MJ/kg dry mass) (Lee et al., 2012). There are only a few studies reported on chemical (acid or alkali) and thermo-chemical pretreatment of different microalgae species so far with the latter, in general, achieving better results in terms of solubilization/methane yield (Bohutskyi et al., 2014; Solé-Bundó et al., submitted). Similar pretreatments, mostly with NaOH or $Ca(OH)_2$ in a wide range of combinations (0.5 -30% w/w, 15 – 160^{o}C, 10 min – 48 h), were previously tested and reported as effective in breaking ester bonds between lignin and polysaccharides and improving both hydrogen/methane production from a variety of lignocellulosic substrates (Monlau et al., 2013). However, controversial results were also obtained for thermo-chemical pretreatment of microalgae. For example, among

chemical (4 M H_2SO_4 at pH = 2, 4 M NaOH, pH = 10), thermal (120^{o}C for 20 or 40 min) and a combination of the aforementioned pretreatments tested, thermally pretreated (120^{o}C, 40 min) *Chlorella vulgaris* produced the highest methane which was attributed to the formation of inhibitory substances during the chemical and thermo-chemical pretreatments (Mendez et al., 2013). More research is needed to identify/quantify inhibitors to optimize thermo-chemical pretreatment of microalgae.

Another bottleneck of microalgal biomass digestion is significantly lower (~6) than optimum C/N ratio (15-30) (Weiland, 2010) of microalgae which may lead to ammonia toxicity to methanogens (Yen and Brune, 2007). One remedy to this problem is co-digestion of microalgal biomass with commonly available, carbon-rich substrates such as paper waste (Yen and Brune, 2007) or lignocellulosic waste (i.e. wheat straw, sorghum, maize) (Rétfalvi et al., 2016). Paper and lignocellulosic wastes can also benefit from moisture and nutrient content of microalgae when co-digested. ~~To the best of our knowledge, lignocellulosic wastes, as co-substrates for microalgae digestion, have not been explored before.~~ If a low-cost pretreatment method, effective for both microalgae and lignocellulosic waste, could be identified, co-digestion of pretreated microalgae and/or the co-substrate could enhance both the rate and extent of digestion with a more favorable energy balance. Therefore, the main objective of this study was to evaluate thermo-alkaline pretreatment of microalgae with wheat straw under both batch and semi-continuous flow mesophilic anaerobic digestion. Thermo-alkaline pretreatment (10% CaO, 72^{o}C, 24 h) was selected based on the previous literature that optimized pretreatment conditions for microalgal biomass digestion (Solé-Bundó et al. submitted). Although these conditions

were optimized for microalgae, literature review indicated that these conditions were also found effective for wheat straw pretreatment (Monlau et al., 2013).

2. Materials and Methods

Batch experiments were conducted at INRA –LBE (Narbonne, France), while semi-continuous flow reactors were operated at GEMMA – UPC (Barcelona, Spain). This necessitated changes in characteristics of inoculum and analytical methods which are outlined below.

2.1. Biochemical methane potential (BMP) assays

2.1.1. Microalgal biomass and lignocellulosic biomass

Microalgal biomass was grown in a pilot-scale high-rate algal pond (HRAP) equipped with a paddle wheel for mixing and had an effective volume of 470 L. HRAP was located outdoors at the laboratory of the GEMMA research group and utilized natural sunlight. The domestic wastewater was first treated in a primary settling tank (effective volume of 7 L, HRT of 0.9 h) and then fed to HRAP under an HRT of 8 days. Upon treatment, effluent from HRAP was sent to a secondary clarifier (9 L, HRT of 9 h) where microalgal biomass was harvested. In order to increase TS concentration to around 2.8 ± 0.1% TS (w/w), microalgal biomass was further thickened in bench-scale Imhoff cones at 4^{o}C for 24 h. Microscopic examination of biomass indicated that the predominant microalgae specie was *Chlorella sp*. although *Monoraphidium sp.* and diatoms were also observed (Fig. 1).

Wheat straw, grown in France (48°50´18´´N, 4°13´54.5´´E), was used as lignocellulosic agricultural biomass. It was processed using a cutting mill, and was further sieved to have a particle size range of 400 µm - 1 mm. Wheat straw characteristics are given in Table 1.

133 *2.1.2. Anaerobic inoculum*

134 The inoculum used was granular sludge from a mesophilic upflow anaerobic sludge blanket
135 (UASB) reactor treating wastewater from a sugar factory in France. Prior to setting up
136 BMP assays, the inoculum was placed in a 5 L glass closed vessel and mixed to break apart
137 the granules under endogenous anaerobic conditions (35^{o}C for 5-7 days) to reduce non-
138 specific biogas generation. The inoculum contained TS and VS concentrations of 2.93 ±
139 0.04 and 2.55 ± 0.03% (w/w), respectively. It had a maximum specific methanogenic
140 activity of 33 ± 2 mL CH_4/g VS/d, as measured by degrading 1.3 ± 0.3 g/L of ethanol as
141 chemical oxygen demand (COD).

142 *2.1.3. Thermo-alkaline pretreatment*

143 Thermo-alkaline pretreatment of microalgal biomass and wheat straw was conducted in
144 glass BMP bottles, with total and effective volumes of 160 and 100 mL, respectively.
145 Microalgal biomass and/or wheat straw were first added to the bottles according to Fig. 2.
146 The bottles were sealed with septa/aluminum caps and kept in an oven (set to 72^{o}C) for 24
147 h without mixing after addition of CaO in dry form (10 g CaO/100 g TS of substrate).
148 Distilled water was added in different amounts to bottles to ensure that all pretreatments
149 were performed at the same TS concentration.

150 *2.1.4. BMP assay set-up*

151 BMP assays were conducted in the same bottles as the thermo-alkaline pretreatment. Upon
152 completion of thermo-alkaline pretreatment, the bottles were cooled down to ambient
153 temperature (~20^{o}C), and the pH of the substrates in the bottles were measured. In order to
154 prevent accumulation of volatile fatty acids (VFAs) during digestion, each bottle was added

5.2 ml of buffer solution prepared at 2.6 g $NaHCO_3$/L concentration. To be able to see the effect of C/N ratio balancing in the co-digested BMPs, the assays were conducted without external nutrient addition. However, considering the risk of not being able to digest wheat straw without nutrient addition, additional bottles were set-up with wheat straw (WS)/ pretreated wheat straw (WS_p) and 1.7 ml of NH_4Cl solution at 0.5 g/L concentration as controls (WS+NH_4Cl and WS_p+NH_4Cl in Fig. 2).

A total of 39 bottles (including triplicates and blanks) were operated to assess the BMP performance (Fig. 2). Each bottle contained substrate (single or co-substrates) concentration of 4 g VS/L. The amount of the substrate and inoculum added to each bottle was calculated considering the food/microorganism (F/M) ratio of 1 gVS/gVS. In the co-digested BMP bottles displayed in Fig. 2, 20, 50 and 80% represented VS weight percentages of microalgal biomass or wheat straw in the total substrate concentration (i.e. 4 g VS/L) in the bottles. Finally, the bottles were filled up to 100 mL with distilled water and nitrogen gas was purged to each bottle to remove residual oxygen. Upon sealing the bottles with septa/caps, the excess pressure caused during the purging was released by puncturing the septa with a needle. The digesters were then located on a shaker (at 90 rpm) in a temperature controlled room at 37°C. Accumulated gas pressure in the bottles was measured with a digital manometer (LEO 2, Keller, Switzerland), while biogas composition was analyzed by a gas chromatograph (GC). In addition to the 39 BMP assays described above, an additional 10 bottles (for 5 pretreatment scenarios in Fig. 2, including duplicates) were initially set-up but sacrificed after pretreatment for characterization of substrates.

2.2. Semi-continuous flow digestion

177 *2.2.1. Microalgal and lignocellulosic biomass*

178 Microalgal biomass was obtained from the same HRAP system described for BMP assays
179 (section 2.1.1) and thickened using the same methodology. Throughout the operation of the
180 semi-continuous flow digesters, TS and VS concentrations of microalgal biomass changed
181 in ranges of 2.6-3.0% and 1.8-2.4%, respectively. The lignocellulosic substrate had
182 identical characteristics described for BMP assays (section 2.1.2). Microalgae and wheat
183 straw were co-digested by 50-50% on VS basis, according to previous BMP assay results.

184 *2.2.2. Anaerobic inoculum*

185 Anaerobic mesophilic digested sludge from a municipal WWTP (Barcelona, Spain) was
186 used to inoculate the semi-continuously fed digesters. The inoculum contained TS and VS
187 concentrations of 2.14 ± 0.01 and 1.31 ± 0.01% (w/w), respectively.

188 *2.2.3. Thermo-alkaline pretreatment*

189 Thermo-alkaline pretreatment of microalgal biomass and wheat straw was conducted
190 together in the same glass bottle, with total and effective volumes of 250 and 150 mL,
191 respectively. Microalgal biomass and/or wheat straw were added to the bottles according to
192 Fig. 2. The bottles were kept in an oven (set to 72^{o}C) for 24 h under continuous stirring
193 after addition of CaO in dry form (10 g CaO/100 g TS of substrate). Distilled water was
194 added in different amounts to bottles to ensure that all pretreatments were performed at the
195 same TS concentration.

196 *2.2.4. Reactor set-up*

197 Microalgae anaerobic digestion performance was monitored using three bench-scale
198 reactors (2 L), with an effective volume of 1.5 L. One of the digesters utilized untreated

199 microalgal biomass and operated as control. The second one simulated a co-digester and
200 received untreated microalgae and wheat straw. The third reactor was fed with thermo-
201 alkaline pretreated microalgal biomass and wheat straw

202 Reactors were operated under mesophilic conditions (37 ± 1^{o}C) by implementing an
203 electric heating cover (Selecta, Spain). Constant mixing was provided by a magnetic stirrer
204 (Thermo Scientific). Reactors were operated on a daily feeding basis, where the same
205 volume was purged from and added to digesters using plastic syringes (50 mL). Reactors
206 were operated at an HRT of 20 days and were considered to be under steady-state after
207 three complete HRTs. Afterwards, anaerobic digestion performance was further monitored
208 during 2 complete HRTs (~6 weeks). The total operation period of the digesters was 106
209 days. Biogas production was measured by the water displacement method and the methane
210 content was periodically analyzed by GC. The volume of the produced biogas was adjusted
211 to the standard temperature (0°C) and pressure (1 atm) condition (STP).

212 **2.3. Analytical procedures**

213 The TS/VS analysis was done according to the Standard Methods (APHA, 2005).
214 Quantification of total and soluble (< 0.45 µm) COD concentrations were performed
215 according to the closed reflux colorimetric method outlined by Standard Methods (APHA,
216 2005). Except for the raw wheat straw samples, all pretreated and untreated substrates and
217 co-substrates were freeze dried (for a minimum of 3 days, at -69°C, 0.25 atm) before
218 structural carbohydrates, lignin, protein and lipid content quantification. Determination of
219 cellulose, hemicelluloses and Klason lignin in raw/pretreated wheat straw were measured
220 using a strong acid hydrolysis method adapted from Sluiter et al. (2008). Raw or freeze-

dried samples (100 mg) were first hydrolyzed with H_2SO_4 (72%) in capped/mixed test tubes at 30°C for 1 h, then diluted to reach a final acid concentration of H_2SO_4 (4%) and kept at 120°C for 1 h. Upon cooling, the tube content was filtered via glass-fiber filters (0.45 µm) to separate insoluble residue, which was placed in a crucible/dried at 100°C for 24 h to yield Klason lignin content. The liquid fraction obtained after filtration was further filtered via 0.2 µm and analyzed by a high-performance liquid chromatograph (HPLC) equipped with a refractive index detector (Waters R410/Waters 2414) for structural carbohydrates (i.e. glucose, xylose and arabinose). Target compounds were separated by an Aminex HPX-87H column (300 x 7.8 mm, Bio-Rad) placed after a protective precolumn (Microguard cation H refill catbridges, Bio-Rad). The eluting solution was 0.005 mM H_2SO_4, and the flowrate, column/detector temperatures were 0.3 mL/min, 45^{o}C, respectively. TKN was determined by titration after a mineralization step performed by a BUCHI 370-K distillator/titrator. Total organic carbon (TOC) was measured using an automatic analyser (aj- Analyzer multi N/C 2100S). TOC was analyzed with an infrared detector (NDIR) according to combustion-infrared method of Standard Methods (APHA, 2005) by means of catalytic oxidation at 800^{o}C using CeO_2 as catalyst. The concentration of the ammonium nitrogen ($N\text{-}NH_4^+$) was measured according to the method by Solorzano (1969). pH was determined with a Crison Portable 506 pH-meter.

Biogas composition in BMP bottles was conducted by measuring the percentage of methane, oxygen, nitrogen, hydrogen, and carbon dioxide in the digester headspace using a GC (Clarus 580, Perkin Elmer) equipped with a thermal conductivity detector (TCD) and RtQBond/RtMolsieve columns. The carrier gas was argon and injector/detector/oven

243 temperatures of 250, 150, 60°C, respectively. Methane percentage from semi-continuous-
244 flow reactors were quantified twice a week with a similar GC/TCD configuration (Trace
245 GC Thermo Finnigan with Hayesep packed column) with injector/detector/oven
246 temperatures were 150, 250, 35°C, respectively, using helium gas as carrier.

247 Volatile fatty acids (VFA) concentrations in semi-continuous flow digesters were measured
248 once a week by injecting 1 µL of each sample, once centrifuged (4200 rpm for 8 min) and
249 filtered (0.2 µm), into an Agilent 7820A GC after sulphuric acid and diisopropyl ether
250 addition. The GC was equipped with an auto-sampler, flame ionization detector and a
251 capillary column (DP-FFAB Agilent 30 m x 0.25 mm x 0.25 µm), and operated at injector
252 and detector temperatures of 200 and 300°C, respectively, with helium as carrier gas.

253 **2.4. Statistics and kinetic data analysis**

254 The statistically significant effects of independent variables were evaluated via multi-factor
255 analysis of variance (ANOVA) considering 95% confidence level (α = 0.05) using R
256 Statistics Software.

257 In order to evaluate the kinetics of the process from BMP tests, experimental data was
258 adjusted to a first-order kinetic model [Eq.1] by the least square method.

259 $B = B_0 \cdot \{1 - exp[-k \cdot t]\}$ [Eq.1]

260 where, B_0 stands for the methane production potential (ml CH_4/gVS), k is the first order
261 kinetic rate constant (day^{-1}), B is the accumulated methane production at time t (ml
262 CH_4/gVS) and t is time (day).

263 The error variance (s^2) was estimated by the following equation [Eq.2]:

264 $s^2 = \frac{\sum_1^i (y_i - \hat{y}_i)^2}{N-K}$ [Eq.2]

where y_i is the experimental value, $\hat{y}_i$ is the value estimated by the model, N is the number of samples and K is the number of model parameters.

3. Results and Discussion

3.1. Thermo-alkaline pretreatment of microalgae and wheat straw

Several studies have recommended the application of pretreatments on microalgae and wheat straw in order to enhance their bioconversion into methane. While microalgae resistant cell wall can be damaged by different pretreatment methods (Passos et al., 2014), lignocellulosic biomass delignification followed by hemicelluloses and cellulose hydrolysis can also be enhanced by applying pretreatments (Croce et al., 2016). Therefore, a thermo-alkaline pretreatment with CaO was tested on both substrates before their anaerobic digestion/co-digestion. The simultaneous application of a pretreatment on both substrates may reduce the operation costs and ease their management in full-scale plants. The pretreatment conditions were 10% CaO at 72^{o}C for 24 h, based on a previous study that evaluated the addition of different CaO doses at different temperatures on microalgae (Solé-Bundó et al., submitted). The study concluded that these conditions lead to the highest levels of carbohydrate and protein solubilization (up to 32 and 31%, respectively). Moreover, 25% methane yield increase compared to untreated microalgae was obtained in BMP tests (Solé-Bundó et al., submitted). In contrast, the methane yield increase achieved by the thermo-alkaline pretreatment in the present study was 9% (Table 2). Although the methane yield of raw microalgae was similar in both cases (260 ml CH_4/g VS in Solé-Bundó et al. and 264 ml CH_4/g VS in this study), the methane yield achieved after applying the same pretreatment was slightly lower in the latter (325 ml CH_4/g VS *vs.* 287 ml CH_4/g

287 VS). This difference may be attributed to the characteristics of the microalgae culture. In
288 the first one the mixed culture was predominated by *Chlorella* sp. and *Scenedesmus* sp.,
289 while in the second one it was mainly predominated by *Chlorella* sp. and contained some
290 diatoms and *Monoraphidium* sp.. It is well known that the methane production from
291 microalgal biomass is highly species-dependent, and not only governed by its biochemical
292 composition but also by their cell structure (Bohutskyi et al., 2014). Comparing the effect
293 of this pretreatment with that obtained by applying other technologies or methods, a
294 moderate effect was here observed. For example, Passos et al. (2015) reported 72%
295 methane yield increase by applying a thermal pretreatment at 95^{o}C for 10 h. Similarly, an
296 enzymatic pretreatment with carbohydrolase and protease showed 55% methane production
297 enhancement on *Chlorella vulgaris* (Mahdy et al., 2014). Although 9% methane yield
298 increase would not justify the pretreatment costs, an important first-order kinetic constant
299 increase was obtained after the pretreatment (from k = 0.085 to 0.133 day^{-1}). This can have
300 an impact on the continuous anaerobic digestion typically operated at 20-30 days of HRT.
301 Compared to microalgae, wheat straw showed a slightly higher methane yield (279 ml
302 CH_4/g VS) but considerably slower kinetics (k = 0.045 day $^{-1}$) (Table 2). Since wheat straw
303 has a very high C/N ratio (~95), the deficit of nitrogen may actually limit the final methane
304 yield obtained in BMPs. Thus, the same wheat straw supplemented by NH_4Cl was also
305 tested (Table 2). When both BMP assays were compared, results showed no significant
306 differences between the methane yields (p-value= 0.926). Concerning the kinetics, when
307 NH_4Cl was added, only a slight increment in the first-order kinetic constant was obtained
308 (from k = 0.045 day^{-1} to 0.049 day^{-1}). This suggests that microorganisms were in fact using

309 the nitrogen from the digested sludge used as inoculum. Therefore, the methane yield of the
310 wheat straw itself was not underestimated, and wheat straw without NH_4Cl could be used
311 as control for the co-digestion analysis in the following sections.
312 Conversely to microalgae, the pretreatment conditions used in this study were not
313 optimized for wheat straw. However, according to Carrere et al. (2015), alkaline
314 pretreatments are promising techniques to enhance the anaerobic digestion of
315 lignocellulosic biomass. Indeed, the application of these pretreatments and their effects
316 have extensively been reported. The main idea is to increase the accessibility and solubility
317 of cellulose and hemicelluloses by facilitating delignification. According to the literature,
318 wheat straw is characterized by having high carbohydrate polymer content (cellulose and
319 hemicelluloses) and relatively low lignin content (Croce et al., 2016). The wheat straw used
320 in this study was composed by 32% cellulose, 29% hemicelluloses and 23% lignin. This
321 composition is coherent with the literature (Barakat et al., 2015). In order to study the effect
322 of the pretreatment on the wheat straw structure, its chemical composition was evaluated
323 before and after pretreatment (Table 1). Slight lignin removal (9%) and more notorious
324 hemicelluloses removal (25%) were observed. Consequently, an increase of soluble sugars
325 was also observed (from 2.8 to 8.4%). However, the celluloses content was not reduced.
326 This is in accordance with most of the literature that evaluated the effect of an alkaline or
327 thermo-alkaline pretreatment on lignocellulosic biomass. However, the level of
328 delignification or hemicelluloses removal varies among them. For instance, Reilly et al.
329 (2015) applied 7.4% of $Ca(OH)_2$ for 42 h to wheat straw obtaining low delignification but
330 30% hemicelluloses removal. On the other hand, Sambusiti et al. (2013) applied 10%

NaOH at 100^{o}C on wheat straw and obtained a higher decrease of lignin (53%). Considering these results, it can be concluded that $Ca(OH)_2$ is not as effective as NaOH, although the pretreatment effectiveness also depends on the substrate. Furthermore, the application of temperature during the pretreatment may facilitate delignification. For example, Monlau et al. (2012) achieved up to 30% lignin removal by applying 4% $Ca(OH)_2$ at 55^{o}C for 24 h on sunflower stalks. Although sunflower stalks composition is similar to that of wheat straw, higher lignin removal was achieved by applying the pretreatment on stalks.

Regarding the methane yield, BMP assays showed 9% increase for pretreated wheat straw compared to the untreated substrate. This is a moderate increase as compared to other studies on alkali pretreatment of lignocellulosic substrates. For example, Monlau et al. (2012) reported 26% increase by pretreating sunflower stalks with 4% $Ca(OH)_2$ at 55^{o}C for 24 h. And significantly higher values (67% increase) were obtained by Sambusiti et al. (2013) by pretreating wheat straw with 10% NaOH at 100^{o}C. Nevertheless, the kinetics were clearly accelerated when the pretreatment was applied (*k* constant increased from 0.045 to 0.122 day^{-1}) (Table 2). Kinetics improvement for pretreated wheat straw was even higher than for pretreated microalgae, especially during the first 50 days of the assay, as it can clearly be seen in Fig. 3a. This can indeed improve the bioconversion process in continuous reactors, so that higher efficiencies could be obtained. Moreover, the application of this pretreatment when microalgae and wheat straw are co-digested should present more benefits than when these substrates are digested alone due to their complementary characteristics.

3.2. Co-digestion performance in BMP tests

Microalgal biomass is characterized by its high nitrogen content, which can limit the substrate utilization during anaerobic digestion. On the contrary, wheat straw mono-digestion can present a deficit of nitrogen due to its high C/N ratio. For that reason, wheat straw has traditionally been co-digested with nitrogen-rich manures (Liu et al., 2015), since both substrates can be easily found in agricultural areas. However, microalgae biomass is an emerging source that offers an alternative for co-digestion with carbon-rich substrates. Therefore, anaerobic co-digestion of microalgae and wheat straw can perform better than the individual anaerobic mono-digestion performances. To evaluate this, the anaerobic co-digestion of three different mixtures of microalgae and wheat straw was compared in BMP assays: 80-20%, 50-50% and 20-80% of microalgae and wheat straw, respectively (VS basis) (Table 2; Fig. 3b). According to section 3.1., the simultaneous pretreatment of both substrates should enhance their anaerobic co-digestion, especially the kinetics. Thus, the same proportions were also tested with pretreated substrates (Table 2; Fig. 3b). The C/N ratios resulting from the mixtures are shown in Table 2. Whereas the mixture with 20% wheat straw still presented a low ratio (C/N= 9), the other proportions (50 and 80% wheat straw) showed values close to 15-30 (C/N= 13 and 26, respectively), suggested as optimal for anaerobic digestion (Weiland, 2010).

The existence of synergies due to co-digestion can be studied by means of BMP tests. BMPs can show whether the final methane yield of the mixtures is actually higher than the methane yield expected as the sum of the methane yield of each substrate (mono-digestion) and / or whether the kinetics improve when the substrates are co-digested. In order to

375 determine if the kinetics of the process was improved by the co-digestion, the first-order
376 kinetic constant was calculated according to Eq. 1 for the BMP curves obtained with the co-
377 digestion (Fig. 3b) and for the expected curves calculated with the values obtained from the
378 mono-digestion of each substrate (data not shown). Both the ultimate methane yield and
379 first-order kinetic constant are reported in Table 2. As can be observed, almost all the
380 experimental methane yields obtained with co-digestion were slightly higher than those
381 expected from the mono-digestion calculations (1-6% methane yield increase). Since this
382 slight increase is similar to BMB assay systematic error (~5%), no conclusive results can be
383 stated regarding the final methane yield increase. In fact, most of the studies that have
384 analyzed the co-digestion of different substrates in BMP assays did not find significant
385 methane yield increase (Astals et al., 2014; Neumann et al., 2015). Moreover, in the studies
386 that did report a methane yield increase, the values obtained were relatively low. For
387 instance, Schwede et al. (2013a) reported about 7% and 9% increase when the marine
388 microalga *Nannochloropsis salina* was co-digested with corn silage and corn-cob-mix,
389 respectively. Nevertheless, the main consistent finding among these studies is that the
390 process kinetics was improved (Astals et al., 2014; Neumann et al., 2015; Ramos-Suárez et
391 al., 2014). Indeed, kinetics improvement was also observed in this experiment by
392 comparing the first-order kinetic constants (Table 2). The highest increase (31%) was found
393 with the highest proportion of wheat straw when the pretreatment was not applied, since it
394 showed a slower degradation.
395 In order to provide an insight into the kinetics analysis, a comparison was made between
396 the methane yield increase of the BMPs with co-digestion and the expected values from the

BMPs with single substrates (mono-digestion) over time (Fig. 4). This figure shows how the methane yield increases were significant during the early days of the experiment. However, when the substrates were not pretreated, synergies could be observed for more than 75 days, with methane yield increases up to 25% for around 14 to 29 days (Fig. 3a). As far as pretreated substrates are concerned, this effect became insignificant after 6 days (Fig. 3b). These results suggest that synergies due to co-digestion took place in both cases, but it was less significant when the biomass was pretreated. This can be attributed to the fact that the pretreatment itself significantly accelerates the kinetics of the process, so the effects of the co-digestion are less discernible than for untreated biomass. Finally, significant differences among substrate proportions could also be observed with untreated substrates. Higher improvements were observed with 50 and 80% wheat straw, corresponding to C/N ratios of 13 and 26, respectively, especially during the first 30 days of assay (Fig. 3). This is in accordance with other studies that found higher synergies when the C/N values were close to 20. For instance, Yen and Brune (2007) suggested an optimum C/N of 20-25 for the co-digestion of algal sludge and waste paper, and Hassan et al. (2016) reported the C/N of 20 for co-digestion of wheat straw and chicken manure. However, no significant differences in methane yield increase were found among C/N ratios when biomass was pretreated.

3.3. Semi-continuous anaerobic co-digestion of microalgae and wheat straw

Co-digestion of 50-50% VS of microalgal biomass and wheat straw was thereafter tested in laboratory-scale semi-continuous reactors. This proportion corresponds to the lowest quantity of wheat straw required to obtain the highest synergistic impact on the co-

digestion, according to the results obtained in the BMP assay. The co-digestion was simultaneously performed for both untreated (digester 2) and pretreated biomass (10% CaO, 72^{o}C, 24 h) (digester 3). Also, a reactor treating microalgal biomass as sole substrate was performed as control (digester 1). During the whole experimental period, all reactors were operated with an organic loading rate (OLR) around 1 g VS/L·day and an HRT of 20 days (Table 3). Weekly average methane yield from each reactor during the steady state period is shown in Fig. 5.

The methane yield of untreated microalgal biomass was 0.12 L CH_4/g VS, with a VS removal around 25%. When microalgae were co-digested with wheat straw, the methane yield increased to 0.21 L CH_4/g VS (77% increase), with a VS removal around 36%. In fact, the methane production rate and yield were significantly higher for the co-digestion reactor in comparison with the control (Table 3). Bearing in mind that the BMP of untreated microalgae and wheat straw were similar, and that the kinetics of the wheat straw was significantly lower than that of microalgae, advantageous results were obtained with their co-digestion in semi-continuous flow. One of the explanations in agreement with literature is the C/N balance achieved by the co-digestion. However, there are other benefits of the co-digestion that can improve the bioconversion process. For instance, Yen and Brune (2007) demonstrated that the co-digestion of algal sludge with waste paper increased the cellulose activity of the digester as compared to the individual algal sludge digestion. On the other hand, Tsapekos et al. (2017) also demonstrated that the co-digestion of manure and lignocellulosic biomass modified and increased the methanogenic activity in the reactor as compared to manure mono-digestion. With regards to pretreated substrates, their co-

digestion showed the best performance with a methane yield of 0.24 L CH_4/g VS and a VS removal around 49%. This represents 102% methane yield increase with respect to microalgae mono-digestion and 15% increase compared to the untreated substrates co-digestion (Table 3).

Concerning the stability of digesters, pH values were stable during the whole period, ranging from 7.2 to 7.5 (Table 3). Although a high pH value (pH=12) of the pretreated effluent was obtained as a consequence of the CaO addition, the pH in digester 3 was nearly neutral (pH = 7.5). Therefore, a good buffer capacity of the digester and substrate dilution may have enabled the operation of the digester without the necessity of externally adjusting the pH. The same fact was reported by Monlau et al. (2015) for continuously-fed digesters with an alkaline pretreated substrate at pH=11 at a similar OLR (1.5 g VS/L·day). Regarding the ammonium concentration, the highest value was observed in the digester treating microalgae as sole substrate. The reactor effluent exhibited around 300 mg N-NH_4/L, which is below toxic concentrations of 1.7 g/L (Schwede et al., 2013b). This is due to the fact that reactors were operated under a very low OLR. In case of increasing this OLR, the ammonium and ammonia concentrations in the reactor would increase and therefore it would have consequences on the stability of the digester. Nevertheless, when wheat straw was added, the ammonium concentration decreased around 2-fold for the untreated substrates and 1.5-fold for the pretreated ones (Table 3). VFAs were not detected in any digester effluent (Table 3). This is again a consequence that the reactors were working at low OLRs and no inhibitions were detected. It is important to highlight that the OLR was fixed by the VS concentrations obtained from low-cost microalgae harvesting

463 (settling and thickening). In fact, Passos and Ferrer (2015) evaluated the anaerobic
464 digestion of microalgae biomass obtained from a similar process and almost no presence of
465 VFAs was detected in the reactors. When wheat straw was added (digesters 2 and 3),
466 dilution of the substrate was necessary to keep the same VS concentrations as the
467 microalgae sole substrate, with the same OLR as the microalgae reactor (digester 1). This
468 allowed for comparison among the three reactors. However, in a full-scale operation, the
469 co-digestion of microalgae with wheat straw could lead to increase the digesters OLR.
470 Overall, the methane yield obtained from microalgae and wheat straw co-digestion, weather
471 pretreated or not, was significantly higher than that obtained from microalgae mono-
472 digestion. By comparing the results from digesters 2 and 3, a low improvement was
473 observed. Only a moderate methane yield increase of 15% was found due to the
474 pretreatment. Although this value is higher than that obtained in the BMP assays (4%), the
475 energy surplus obtained from the methane production increase would not compensate the
476 energy requirements and chemical costs to perform the pretreatment step. Indeed, the study
477 carried out by Passos and Ferrer (2014) concluded that 33% methane production increase
478 was necessary to achieve a neutral energy balance when microalgae biomass was pretreated
479 at 75^{o}C for 10 h. On the contrary, the co-digestion of microalgae and wheat straw presents
480 some advantages. For example, the addition of wheat straw increases the efficiency of the
481 reactor, mainly due to the C/N balance. But also, it allows for an increase in the OLR of the
482 digestion by avoiding the stability problems that microalgae mono-digestion can present
483 (inhibition due to high N-NH_4). For example, Herrmann et al. (2016) demonstrated that
484 while the anaerobic digestion of the microalgae *Arthisoira platensis* was stable at a low

485 OLR of 1 g VS/L·day, their co-digestion with a carbon-rich substrate (brown seaweed)
486 achieved an OLR up to 4 g VS/L·day. Another advantage of co-digesting microalgae and
487 wheat straw without any pretreatment is that the only additional energy required is related
488 to wheat straw milling. In this study, a milled wheat straw between 400 and 1 mm was
489 used. However, for a more efficient performance, an optimization of the milling would be
490 recommended. On the other hand, one of the most limiting costs associated to the co-
491 digestion is the transport of the co-substrates from their origin to the digestion plant (Mata-
492 Alvarez et al., 2014). For that reason, the wheat crop area should be located nearby the
493 digestion plant.

494 **4. Conclusions**

495 This study showed how microalgae and wheat straw co-digestion improved either mono-
496 digestion in BMP assays. Higher improvements ~~The best results~~ were obtained with
497 untreated microalgae and wheat straw mixtures of 50-50% and 20-80%, with C/N ratios of
498 13 and 26, respectively. The co-digestion of 50-50% microalgae and wheat straw in lab-
499 scale reactors increased the methane yield by 77% compared to microalgae mono-digestion,
500 while the pretreatment only increased the methane yield by 15% compared to the untreated
501 substrates co-digestion. Thus, the co-digestion of microalgae and wheat straw was
502 successful even without the thermo-alkaline pretreatment.

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References

Alzate, M.E., Muñoz, R., Rogalla, F., Fdz-Polanco, F., Pérez-Elvira, S.I., 2012. Biochemical methane potential of microalgae: Influence of substrate to inoculum ratio, biomass concentration and pretreatment. Bioresour. Technol. 123, 488–494. doi:10.1016/j.biortech.2012.06.113

Astals, S., Batstone, D.J., Mata-Alvarez, J., Jensen, P.D., 2014. Identification of synergistic impacts during anaerobic co-digestion of organic wastes. Bioresour. Technol. 169, 421–427. doi:10.1016/j.biortech.2014.07.024

Barakat, A., Monlau, F., Solhy, A., Carrere, H., 2015. Mechanical dissociation and fragmentation of lignocellulosic biomass: Effect of initial moisture, biochemical and structural proprieties on energy requirement. Appl. Energy 142, 240–246. doi:10.1016/j.apenergy.2014.12.076

Bohutskyi, P., Betenbaugh, M.J., Bouwer, E.J., 2014. The effects of alternative pretreatment strategies on anaerobic digestion and methane production from different algal strains. Bioresour. Technol. 155, 366–72. doi:10.1016/j.biortech.2013.12.095

Carrere, H., Antonopoulou, G., Affes, R., Passos, F., Battimelli, A., Lyberatos, G., Ferrer, I., 2015. Review of feedstock pretreatment strategies for improved anaerobic digestion: From lab-scale research to full-scale application. Bioresour. Technol. 199, 386–397. doi:10.1016/j.biortech.2015.09.007

Croce, S., Wei, Q., D'Imporzano, G., Dong, R., Adani, F., 2016. Anaerobic digestion of straw and corn stover: The effect of biological process optimization and pre-treatment on total bio-methane yield and energy performance. Biotechnol. Adv. 34, 1289–1304. doi:10.1016/j.biotechadv.2016.09.004

Golueke, C.G., Oswald, W.J., Gotaas, H.B., 1957. Anaerobic digestion of Algae. Appl. Microbiol. 5, 47–55.

Hassan, M., Ding, W., Shi, Z., Zhao, S., 2016. Methane enhancement through co-digestion of chicken manure and thermo-oxidative cleaved wheat straw with waste activated sludge: A C/N optimization case. Bioresour. Technol. 211, 534–541. doi:10.1016/j.biortech.2016.03.148

Herrmann, C., Kalita, N., Wall, D., Xia, A., Murphy, J.D., 2016. Optimised biogas production from microalgae through co-digestion with carbon-rich co-substrates. Bioresour. Technol. 214, 328–337. doi:10.1016/j.biortech.2016.04.119

543 Lee, A.K., Lewis, D.M., Ashman, P.J., 2012. Disruption of microalgal cells for the
544 extraction of lipids for biofuels: Processes and specific energy requirements. Biomass
545 and Bioenergy 46, 89–101. doi:10.1016/j.biombioe.2012.06.034
546 Liu, L., Zhang, T., Wan, H., Chen, Y., Wang, X., Yang, G., Ren, G., 2015. Anaerobic co-
547 digestion of animal manure and wheat straw for optimized biogas production by the
548 addition of magnetite and zeolite. Energy Convers. Manag. 97, 132–139.
549 doi:10.1016/j.enconman.2015.03.049
550 Mahdy, A., Mendez, L., Ballesteros, M., González-Fernández, C., 2014. Enhanced methane
551 production of Chlorella vulgaris and Chlamydomonas reinhardtii by hydrolytic
552 enzymes addition. Energy Convers. Manag. 85, 551–557.
553 doi:10.1016/j.enconman.2014.04.097
554 Mata-Alvarez, J., Dosta, J., Romero-Güiza, M.S., Fonoll, X., Peces, M., Astals, S., 2014. A
555 critical review on anaerobic co-digestion achievements between 2010 and 2013.
556 Renew. Sustain. Energy Rev. 36, 412–427. doi:10.1016/j.rser.2014.04.039
557 Mendez, L., Mahdy, A., Timmers, R.A., Ballesteros, M., González-Fernández, C., 2013.
558 Enhancing methane production of Chlorella vulgaris via thermochemical
559 pretreatments. Bioresour. Technol. 149, 136–41. doi:10.1016/j.biortech.2013.08.136
560 Monlau, F., Barakat, A., Steyer, J.P., Carrere, H., 2012. Comparison of seven types of
561 thermo-chemical pretreatments on the structural features and anaerobic digestion of
562 sunflower stalks. Bioresour. Technol. 120, 241–7. doi:10.1016/j.biortech.2012.06.040
563 Monlau, F., Barakat, A., Trably, E., Dumas, C., Steyer, J.-P., Carrère, H., 2013.
564 Lignocellulosic materials into biohydrogen and biomethane: impact of structural
565 features and pretreatment. Crit. Rev. Environ. Sci. Technol. 43, 260–322.
566 Monlau, F., Kaparaju, P., Trably, E., Steyer, J.P., Carrere, H., 2015. Alkaline pretreatment
567 to enhance one-stage CH4 and two-stage H2/CH4 production from sunflower stalks:
568 Mass, energy and economical balances. Chem. Eng. J. 260, 377–385.
569 doi:10.1016/j.cej.2014.08.108
570 Neumann, P., Torres, A., Fermoso, F.G., Borja, R., Jeison, D., 2015. Anaerobic co-
571 digestion of lipid-spent microalgae with waste activated sludge and glycerol in batch
572 mode, International Biodeterioration & Biodegradation.
573 doi:10.1016/j.ibiod.2015.01.020
574 Passos, F., Carretero, J., Ferrer, I., 2015a. Comparing pretreatment methods for improving
575 microalgae anaerobic digestion: Thermal, hydrothermal, microwave and ultrasound.
576 Chem. Eng. J. 279, 667–672. doi:10.1016/j.cej.2015.05.065
577 Passos, F., Ferrer, I., 2015. Influence of hydrothermal pretreatment on microalgal biomass
578 anaerobic digestion and bioenergy production. Water Res. 68, 364–373.
579 doi:10.1016/j.watres.2014.10.015
580 Passos, F., Ferrer, I., 2014. Microalgae Conversion to Biogas: Thermal Pretreatment
581 Contribution on Net Energy Production. Environ. Sci. Technol. 48, 7171–7178.
582 doi:10.1021/es500982v

583 Passos, F., Gutiérrez, R., Brockmann, D., Steyer, J.-P., García, J., Ferrer, I., 2015b.
584 Microalgae production in wastewater treatment systems, anaerobic digestion and
585 modelling using ADM1. Algal Res. 10, 55–63. doi:10.1016/j.algal.2015.04.008
586 Passos, F., Uggetti, E., Carrère, H., Ferrer, I., 2014. Pretreatment of microalgae to improve
587 biogas production: A review. Bioresour. Technol. 172, 403–412.
588 doi:10.1016/j.biortech.2014.08.114
589 Ramos-Suárez, J.L., Martínez, A., Carreras, N., 2014. Optimization of the digestion process
590 of Scenedesmus sp. and Opuntia maxima for biogas production. Energy Convers.
591 Manag. 88, 1263–1270. doi:10.1016/j.enconman.2014.02.064
592 Rawat, I., Ranjith Kumar, R., Mutanda, T., Bux, F., 2011. Dual role of microalgae:
593 Phycoremediation of domestic wastewater and biomass production for sustainable
594 biofuels production. Appl. Energy 88, 3411–3424.
595 doi:10.1016/j.apenergy.2010.11.025
596 Rétfalvi, T., Szabó, P., Hájos, A.-T., Albert, L., Kovács, A., Milics, G., Neményi, M.,
597 Lakatos, E., Ördög, V., 2016. Effect of co-substrate feeding on methane yield of
598 anaerobic digestion of Chlorella vulgaris. J. Appl. Phycol. 28, 2741–2752.
599 doi:10.1007/s10811-016-0796-5
600 Sambusiti, C., Monlau, F., Ficara, E., Carrère, H., Malpei, F., 2013. A comparison of
601 different pre-treatments to increase methane production from two agricultural
602 substrates. Appl. Energy 104, 62–70. doi:10.1016/j.apenergy.2012.10.060
603 Schwede, S., Kowalczyk, A., Gerber, M., Span, R., 2013a. Anaerobic co-digestion of the
604 marine microalga Nannochloropsis salina with energy crops. Bioresour. Technol. 148,
605 428–435. doi:10.1016/j.biortech.2013.08.157
606 Schwede, S., Rehman, Z.-U., Gerber, M., Theiss, C., Span, R., 2013b. Effects of thermal
607 pretreatment on anaerobic digestion of Nannochloropsis salina biomass. Bioresour.
608 Technol. 143, 505–511. doi:10.1016/j.biortech.2013.06.043
609 Sluiter, A., (U.S.), N.R.E.L. (Eds.), 2008. Determination of sugars, byproducts, and
610 degradation products in liquid fraction process samples [electronic resource] :
611 laboratory analytical procedure (LAP) : issue date, 12/08/2006 / A. Sluiter ... [et al.],
612 NREL/TP ; 510-42623. National Renewable Energy Laboratory, Golden, Colo.
613 Solé-Bundó, M., Cucina, M., Folch, M., Tàpias, J., Gigliotti, G., Garfí, M., Ferrer, I., 2017.
614 Assessing the agricultural reuse of the digestate from microalgae anaerobic digestion
615 and co-digestion with sewage sludge. Sci. Total Environ. 586, 1–9.
616 doi:10.1016/j.scitotenv.2017.02.006
617 Solorzano, L., 1969. Determination of ammonia in natural waters by the
618 phenolhypochlorite method. Limnol. Ocean. 14, 799.
619 Tsapekos, P., Kougias, P.G., Treu, L., Campanaro, S., Angelidaki, I., 2017. Process
620 performance and comparative metagenomic analysis during co-digestion of manure
621 and lignocellulosic biomass for biogas production. Appl. Energy 185, 126–135.
622 doi:10.1016/j.apenergy.2016.10.081

623 Ward, A.J., Lewis, D.M., Green, F.B., 2014. Anaerobic digestion of algae biomass: A
624 review. Algal Res. 5, 204–214. doi:10.1016/j.algal.2014.02.001
625 Weiland, P., 2010. Biogas production: Current state and perspectives. Appl. Microbiol.
626 Biotechnol. 85, 849–860. doi:10.1007/s00253-009-2246-7
627 Yen, H.-W., Brune, D.E., 2007. Anaerobic co-digestion of algal sludge and waste paper to
628 produce methane. Bioresour. Technol. 98, 130–4. doi:10.1016/j.biortech.2005.11.010
629

630 **Table. 1.** Chemical composition of wheat straw, before and after the thermo-alkaline
631 pretreatment. Mean values ± standard deviation of triplicates.

	Wheat straw	**Pretreated wheat straw**
TS (%)	93.5 ± 0.1	94.2 ± 0.9
VS (%)	89.4 ± 0.1	84.8 ± 0.8
VS/TS (%)	95.6 ± 0.0	87.8 ± 0.3
Lignin (%, VS)	23.0 ± 0.4	21.0 ± 0.2
Cellulose (%, VS)	32.5 ± 0.2	32.1 ± 0.6
Hemicellulose (%, VS)	28.8 ± 0.2	21.7 ± 0.2
Soluble sugars[a] (%, VS)	2.8 ± 0.4	8.4 ± 0.0
Acetate (%, VS)	3.8 ± 0.1	3.4 ± 0.2

632 [a] Glucose, xylose, ramnose, arabinose, succinate, glycerol and acetate

Table. 2. Ultimate methane yield obtained in the BMP assay (mean values ± standard deviation; n=3) and first-order kinetics (k) obtained from Eq.1. (the error variance (S^2) of each fitting (Eq. 2) is represented in brackets).

Substrates	**C/N**	**Methane yield**, ml CH_4/g VS				**First-order kinetics,** day^{-1}			
		Experimental values[a]		**Calculated values from mono-digestions**[b]		**Experimental values**[a]		**Calculated values from mono-digestions**[c]	
		Untreated	Pretreated	Untreated	Pretreated	Untreated	Pretreated	Untreated	Pretreated
Control Microalgae	7.4	264 ±3	287 ±9	-	-	0.085 (175)	0.133 (205)	-	-
80% Microalgae + 20% Wheat Straw	8.9	279 ±6	289 ±15	267 ± 3	290 ± 7	0.079 (114)	0.150 (186)	0.075 (199)	0.131 (188)
50% Microalgae + 50% Wheat Straw	13.1	289 ±3	299 ±15	271 ± 5	295 ± 6	0.071 (80)	0.150 (159)	0.062 (224)	0.127 (166)
20% Microalgae + 80% Wheat Straw	26.4	289 ±4	315 ±7	276 ± 7	300 ± 6	0.067 (55)	0.142 (172)	0.051 (236)	0.124 (147)
Control Wheat Straw	95.4	279 ±9	304 ±7	-	-	0.045 (240)	0.122 (136)	-	-
Control Wheat Straw + NH_4Cl	-	280 ±9	303 ±7	-	-	0.049 (61)	0.125 (157)	-	-

[a] Values obtained from experimental data in BMP assay

[b] Values calculated as the sum of the final methane yields produced for each substrate mono-digestion: ((pretreated) wheat straw/(pretreated) microalgae).

[c] Values obtained from the curves that represent the sum of the individual ((pretreated) wheat straw /(pretreated) microalgae) methane yields produced over the time.

Table. 3. Influent and digested biomass characteristics from microalgae semi-continuous anaerobic digestion (control) and co-digestion with wheat straw (50-50% VS), with and without thermo-alkaline pretreatment(10% CaO at 72°C for 24 h). Mean ± standard deviation of 6 samples from steady-state.

Parameter	**Digester 1: Control Microalgae**	**Digester 2: Co-digestion**	**Digester 3: Co-digestion + pretreatment**
Operation conditions			
HRT (days)	20	20	20
OLR (kg VS/m^3 d))	1.12 ± 0.07	1.04 ± 0.03	0.97 ± 0.02
Influent composition			
pH	7.06 ± 0.14	6.82 ± 0.10	12.04 ± 0.18
TS [% (w/w)]	2.74 ± 0.14	2.39 ± 0.14	2.70 ± 0.11
VS [% (w/w)]	2.10 ± 0.10	2.06 ± 0.12	1.97 ± 0.16
VS/TS (%)	79.8 ± 3.0	86.2 ± 1.7	71.9 ± 5.7
C/N (-)	4.7 ± 0.4	13.7 ± 2.1	12.8 ± 2.0
N-NH_4 (mg/L)	28 ± 8	15 ± 5	44 ± 9
Effluent composition			
pH	7.51 ± 0.27	7.17 ± 0.18	7.49 ± 0.16
TS [% (w/w)]	2.32 ± 0.13	1.75 ± 0.06	1.79 ± 0.04
VS [% (w/w)]	1.65 ± 0.08	1.36 ± 0.04	0.98 ± 0.03
VS/TS (%)	70.8 ± 0.9	78.1 ± 1.1	54.5 ± 0.8
N-NH_4 (mg/L)	304 ± 25	160 ± 39	199 ± 59
VFA (mg COD/L)	<LOD	<LOD	<LOD
Removal efficiency			
TS removal (%)	18.0 ± 2.7	33.1 ± 5.1	35.4 ± 1.5
VS removal (%)	26.3 ± 5.2	37.6 ± 2.8	48.3 ± 2.9
Biogas production			
Methane production rate (L CH_4/L·d)	0.14 ± 0.02	0.21 ± 0.03	0.23 ± 0.02
Methane yield (L CH_4/g VS)	0.12 ± 0.02	0.21 ± 0.03	0.24 ± 0.02
Methane content in biogas (% CH_4)	67.8 ± 0.3	61.8 ± 2.1	67.0 ± 0.7

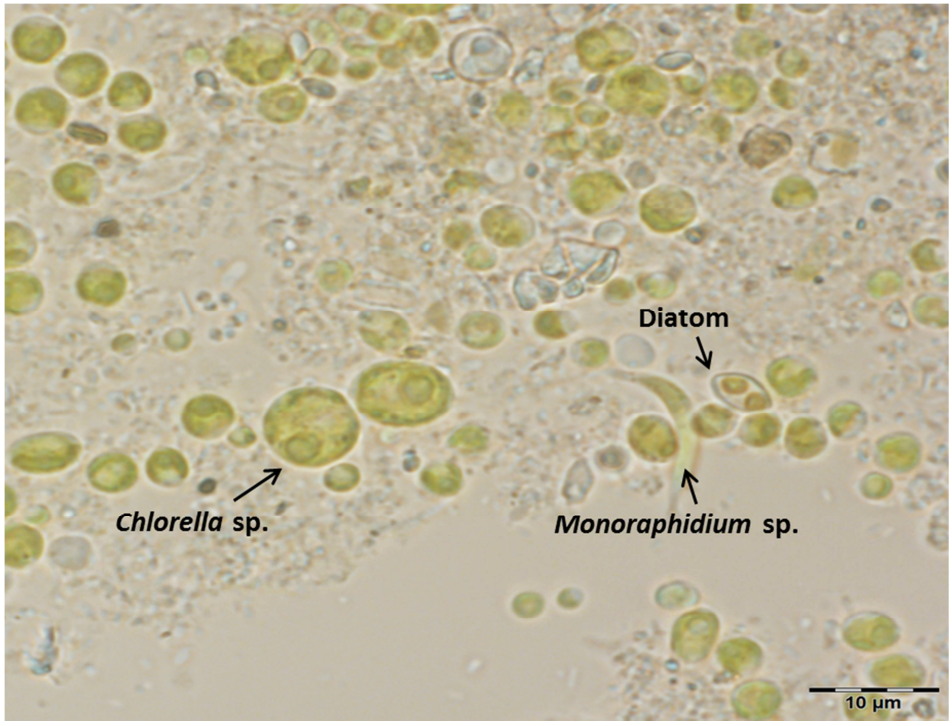


Fig. 1. Microscopic image of microalgal biomass, mainly composed by *Chlorella* sp. although *Monoraphidium* sp. and diatoms were also observed.

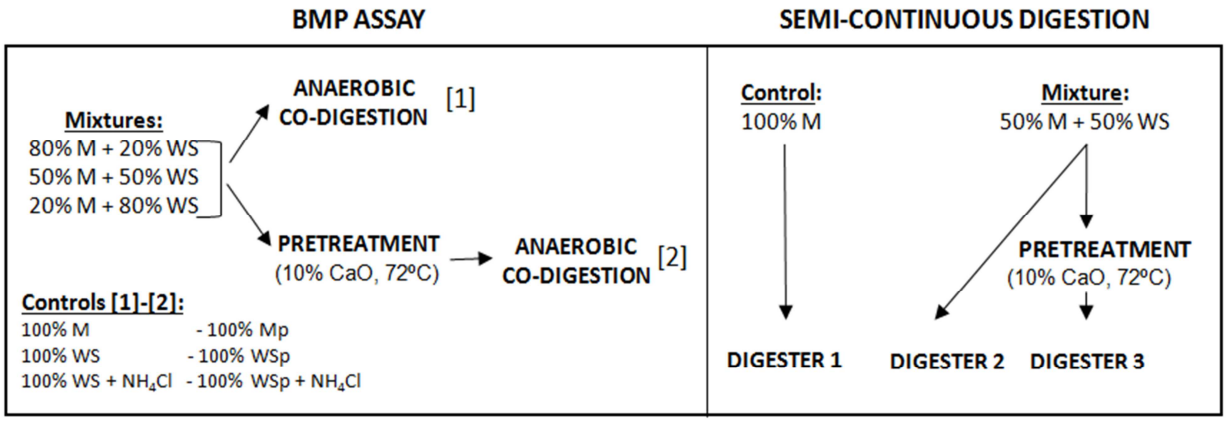
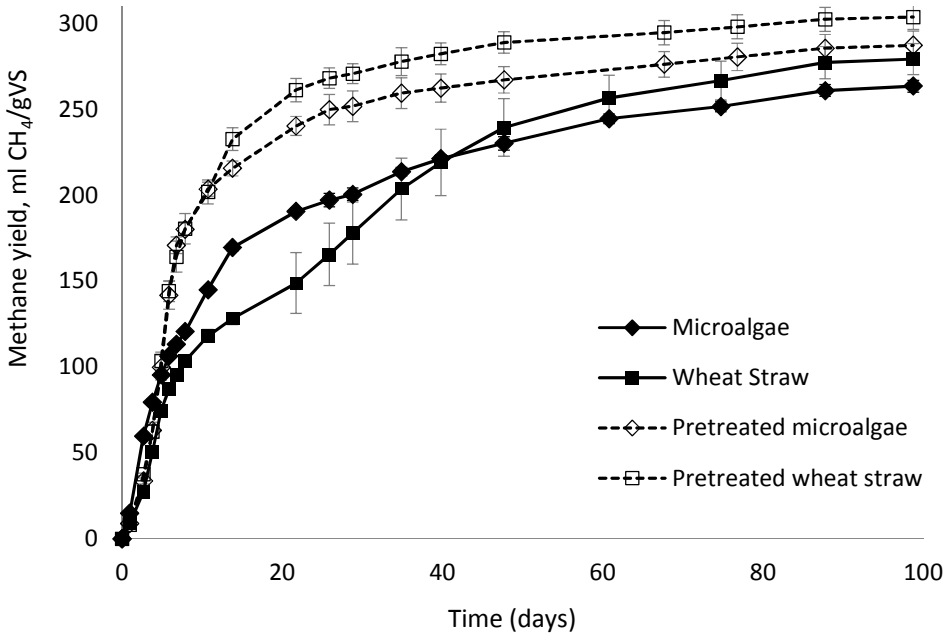
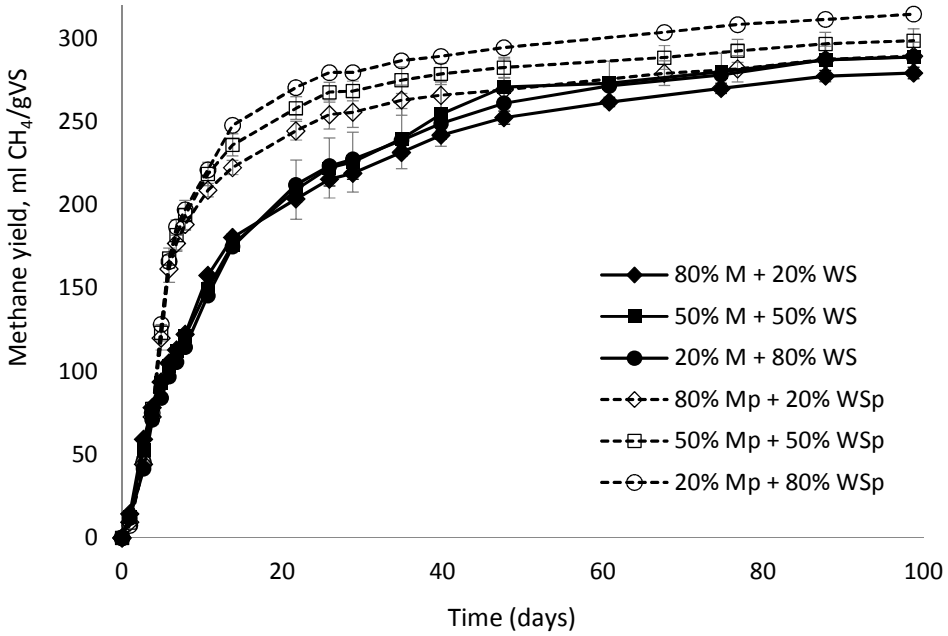


Fig. 2. Experimental set-up.

Note: M= microalgae; Mp= pretreated microalgae; WS= wheat straw; WSp= pretreated wheat straw

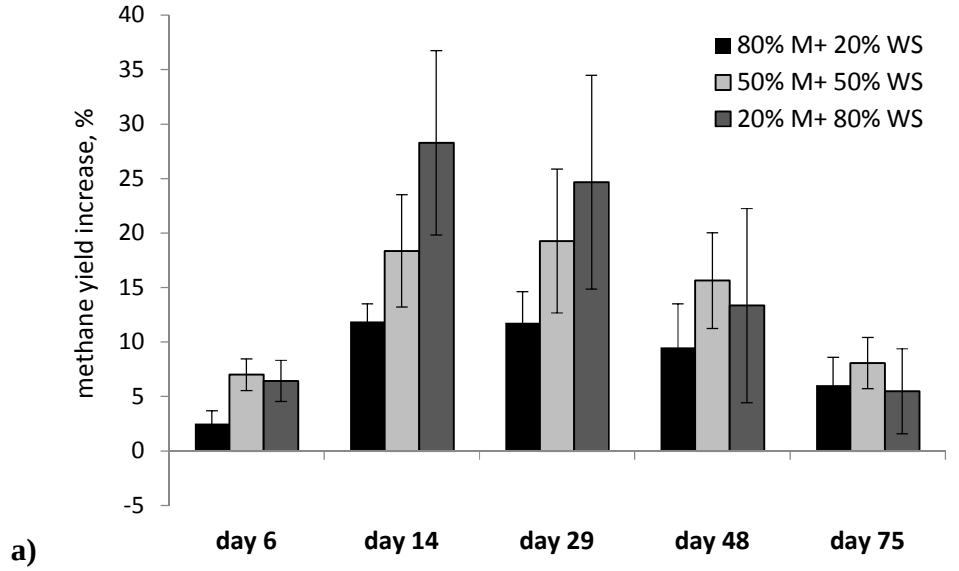


a)

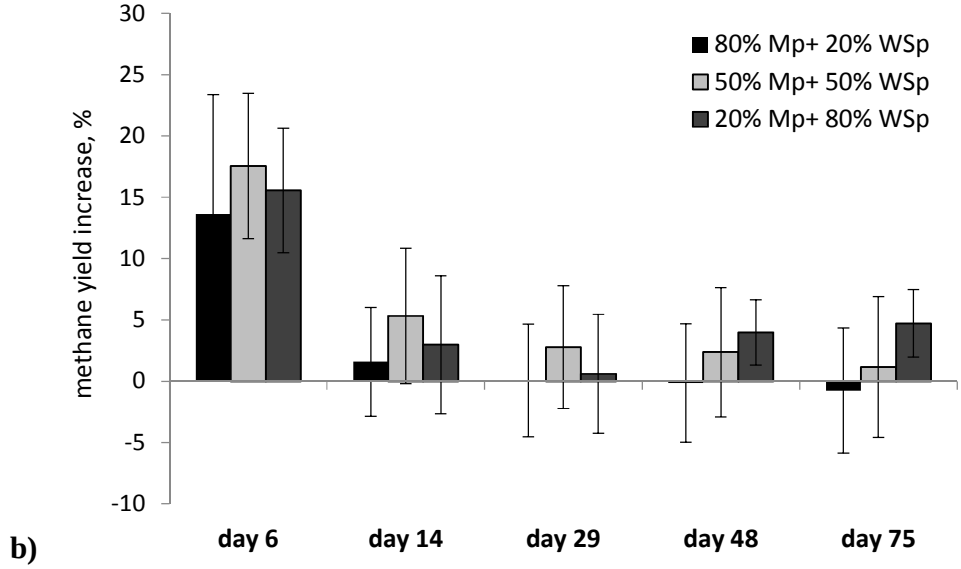


b)

Fig. 3. Cumulative methane yield of raw microalgae and wheat straw (controls) and with a thermo-alkaline pretreatment (10% CaO at 72°C for 24 h) (a) and their anaerobic co-digestion (80-20%VS; 50-50%VS and 20-80%VS, respectively) with untreated and preatreated substrates (b).
Note: M= microalgae; Mp= pretreated microalgae; WS= wheat straw; WSp= pretreated wheat straw



a)



b)

Fig. 4. Methane yield increase of co-digested samples with respect to calculated values proportional to mono-digested substrates (microalgae and wheat straw) without pretreatment (a) and with thermo-alkaline pretreatment (10% CaO at 72°C for 24 h) (b) after 6, 14, 29, 48 and 75 days of BMP assay.

Note: M= microalgae; Mp= pretreated microalgae; WS= wheat straw; WSp= pretreated wheat straw

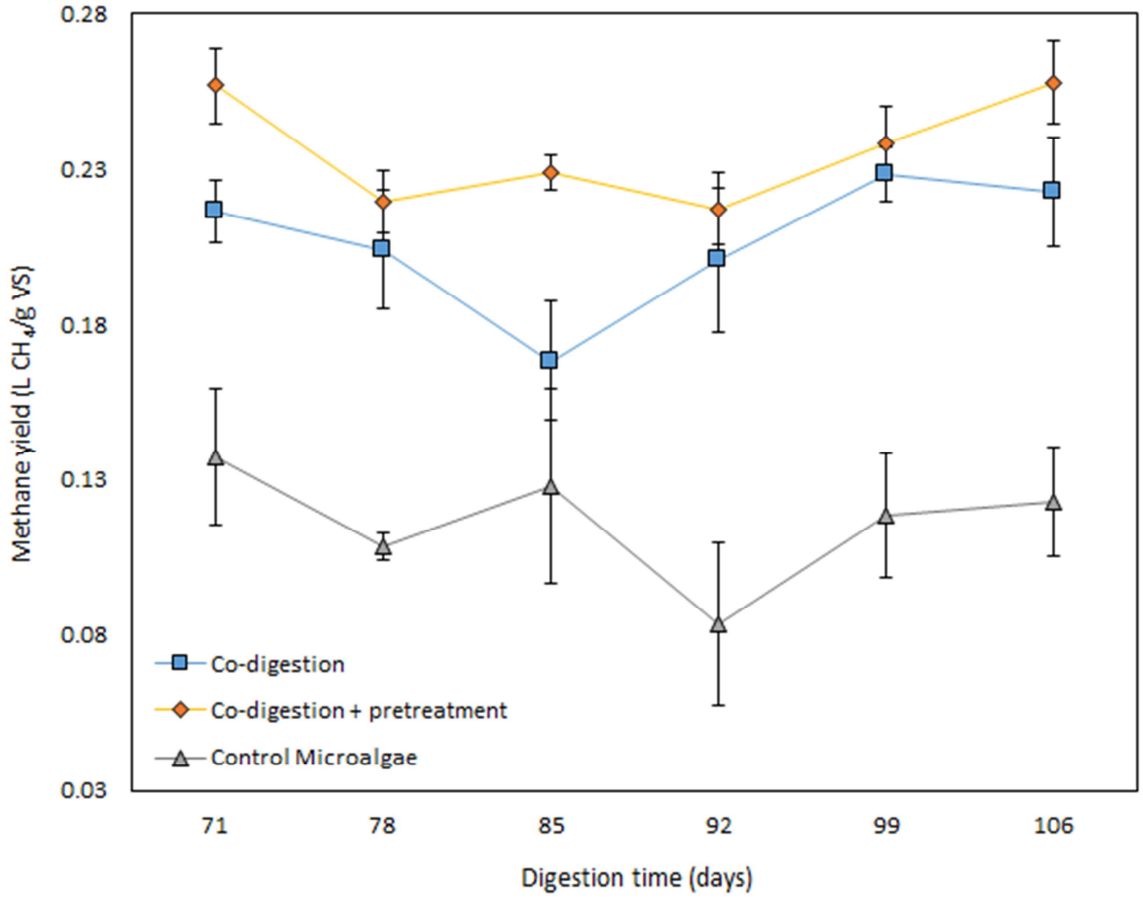


Fig. 5. Steady-state weekly average methane yields of untreated microalgae (control), untreated microalgae and wheat straw co-digestion (50-50%) (co-digestion) and thermo-alkaline pretreated microalge and wheat straw co-digestion (50-50%) (co-digestion+pretreatment) obtained in semi-continuous reactors.