Integrating microalgae tertiary treatment into activated sludge systems for energy and nutrients recovery from wastewater

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Abstract

In this study, microalgae digestate and secondary effluent were used to grow microalgae in a tertiary wastewater treatment, and then, the biomass was co-digested for biogas generation. A 30L closed-photobioreactor was used for microalgae cultivation. The biomass, mainly composed by *Scenedesmus* sp., reached and maintained a concentration of 1.1 gTSS/L during 30 days. A complete removal of $N{-}NH_4^+$ and $P\text{-}PO_4^{3-}$ and high nitrates and organic matter removals were achieved (58 % $N\text{-}NO_3^-$ and 70 % COD) with 8d of HRT. The potential biogas production of the cultivated microalgae was determined in batch tests. To improve their biodegradability, a novel method combining their co-digestion with activated sludge after a simultaneous autohydrolysis co-pretreatment was evaluated. After the co-pretreatment, the methane yield increased by 130 %. Thus, integrating microalgae tertiary treatment into activated sludge systems is a promising and feasible solution to recover energy and nutrients from waste, improving wastewater treatment plants sustainability.

Keywords: anaerobic digestion, autohydrolysis pretreatment, bioenergy, biogas, centrate, microalgal biomass

1. Introduction

Until now, wastewater treatment plants (WWTPs) were mainly conceived for removing contaminants and organic matter, and were designed and managed to protect human and environmental health (Muga and Mihelcic, 2008). However, the increasing water scarcity forces the need for new technological solutions with low cost and low energy demand (Chisti, 2008). To transform a conventional wastewater treatment system into a self-sustainable process it is necessary to shift from the current model towards a new one in which wastewater treatment systems will become a low energy processing industry, able to generate marketable products rather than wastes. For this reason, special efforts have been made recently to increase energy and resource recovery from wastewater by producing valuable byproducts (e.g. biofuels) from WWTPs.

Under this scenario, nature-based treatment solutions, such as microalgae-based systems, are conceived as a breakthrough to a new model for wastewater treatment (Pittman et al., 2011). Indeed, such systems are able to reuse nutrients from wastewater and other wastes (i.e. digestate from anaerobic digestion) in order to grow microalgae biomass which can be used as bioenergy feedstock (Uggetti et al., 2014a). However, the alternative of recycling microalgae digestate has been poorly explored. The main concern in the use of digestate as nutrient for microalgae growth is the elevated ammonium content. Though, this inconvenience may be solved by diluting it with another low strength waste effluent (i.e. secondary effluent from wastewater treatment).

Considering small-medium conventional WWTPs based on the activated sludge process with anaerobic digestion for waste activated sludge (WAS) treatment, a microalgae

65 photobioreactor (PBR) could be introduced as a tertiary treatment in order to improve the
66 treated water quality and increase the biogas production (Figure 1). Indeed, the microalgae
67 biomass produced in the PBR could be co-digested with waste activated sludge from the
68 conventional plant. In such a case, their co-digestion could improve the methane productivity
69 and the hydrolysis efficiency compared to each substrate mono-digestion, increasing the
70 bioenergy recovery efficiency of the plant (Zhen et al., 2016). In fact, recent investigation
71 has reported higher methane yield and/or rate when microalgae and WAS are co-digested
72 (Beltran et al., 2016; Neumann et al., 2015). Besides, WAS has inherent enzymes inside its
73 extracellular polymeric substances (EPS) which are released after a thermal pretreatment at
74 55ºC resulting in autohydrolysis of WAS (Carvajal et al., 2013). Hence, the co-pretreatment
75 and subsequent co-digestion of microalgae and WAS may improve the hydrolysis. Moreover,
76 the digestate from the anaerobic digestion could be reused as a source of nutrients for
77 microalgae biomass growth together with the secondary effluent. In this way, the quality of
78 treated wastewater would be improved, as compared to conventional biological systems, and
79 the digestate would be treated while increasing the concentration of nutrients for microalgae
80 growth.
81
82 Following the scheme proposed in Figure 1, this article addresses a novel approach in the
83 field of wastewater treatment. Previous studies focused on microalgae production for biogas
84 production (i.e., Passos et al., 2015, 2013; Passos and Ferrer, 2014), were addressed to treat
85 urban wastewater by means of high rate algal ponds as a secondary treatment. Differently,
86 this study proposes an integrated system of activated sludge and microalgae tertiary treatment
87 for nutrients and bioenergy recovery from wastewater. Thus, the objectives of this research
88 were: 1) to study the microalgal biomass production treating the secondary wastewater

effluent and digestate; and 2) to quantify the methane yield of harvested microalgae biomass co-digested with waste activated sludge after an autohydrolysis pretreatment.

2. Methodology

2.1 Experimental set-up

Experiments were carried out at the laboratory of the GEMMA Research Group (Barcelona, Spain). Microalgae were grown in a closed cylindrical photobioreactor (30L). The PBR was fed with microalgae uncentrifuged digestate diluted in secondary effluent from a pilot high rate algal pond (HRAP) treating municipal wastewater. The latter came from a pilot system treating municipal wastewater which comprised a primary settler, a high rate algal pond (HRAP) and a secondary settler (Gutiérrez et al., 2016). The digestate was obtained from lab-scale anaerobic digesters (1.5 L) that produced biogas from microalgae biomass harvested from the HRAP. A detailed description of the anaerobic digesters and HRAP may be found in Passos et *al*. (2015).

2.2 Photobioreactor operation

A mixed microalgae culture obtained from a pilot high rate algal pond was utilized as inoculum to start-up the photobioreactor. This inoculum consisted of a community of microalgae, bacteria, protozoa and small metazoan, specifically dominated by the microalgae genus *Chlorella* sp., *Scenedesmus* sp. and *Stigeoclonium* sp. The closed photobioreactor was located indoors and consisted of a cylindrical vessel made of polymethyl methacrylate with a working volume of 30 L. The mixed liquor was stirred by means of an air sparger placed at the bottom of the photobioreactor, at a flow of 10 L/min and a pressure of 0.034 MPa using a 105 W air compressor (model ACQ-012, JAD, China). The photobioreactor design and

operation characteristics may be found elsewhere (Arias et al., 2017). The culture in the photobioreactor was in continuous operation alternating light:dark periods of 12 h. During the illuminance period, light was supplied by an external lamp (600W, Sunmaster, USA) placed at 80 cm in front of the photobioreactor, providing 19,000 lux (289 μmol/m^2s). The temperature of the culture along the experimental period ranged from 25 to 29 ºC.

The photobioreactor was fed once a day (semi-continuously) with microalgae digestate diluted in secondary effluent at a ratio of 1:50, and operated at 8 days of hydraulic retention time (HRT) and solids retention time (SRT). The dilution ratio of 1:50 was performed in order to decrease the ammonium ($N{-}NH_4^+$) content to concentrations below 10 mg/L in the photobioreactor influent. The physico-chemical characterization of the digestate and secondary effluent used as influent for microalgae growth in the photobioreactor is shown in Table 1.

2.3 Biochemical methane potential assay

2.3.1. Substrates and inoculum

The microalgae biomass used in the biochemical methane potential (BMP) assays was collected from the photobioreactor effluent after stable operation. At the time, the microalgae biomass was clearly dominated by *Scenedesmus* sp. Harvested biomass was settled for 1 day, and then thickened for 3h to reach the target total solids (TS) concentration of 2.8 %. This procedure was performed at 5ºC to preserve microalgae properties.

135 WAS was used as co-substrate for *Scenedesmus* sp digestion. It was obtained from a
136 secondary settler of a conventional WWTP (Barcelona, Spain). WAS had a TS and VS
137 content of 1.8 % and 1.3 %, respectively. It was stored at 5 ºC until use.
138
139 Mesophilic digested sludge from the same WWTP (Barcelona, Spain) was used as inoculum
140 for BMP assays and was stored at 5 ºC until use.
141
142 ***2.3.2. Autohydrolysis pretreatment: preliminary solubilisation assay***
143 A preliminary solubilisation assay was carried out in order to determine the optimal contact
144 time for the autohydrolysis pretreatment. The assay was performed at 55 ºC in order to
145 activate WAS enzymes (Carvajal et al., 2013).
146
147 The autohydrolysis pretreatment was carried out in four glass bottles with a total volume of
148 250 mL and liquid volume of 200 ml each. Bottles were placed in a heater under mild
149 continuous mixing using multi magnetic stirrers at a constant temperature of 55 ºC. Trials
150 were prepared with microalgae and WAS alone (controls) and with mixtures of microalgae
151 and WAS at different proportions: 50 % microalgae + 50 % WAS and 80 % microalgae + 20
152 % WAS (on a VS basis).
153
154 Time course of biomass solubilisation was analysed from the solubilisation curves defined
155 by the solubilisation ratio (S) obtained at increasing exposure times. The solubilisation ratio
156 was defined as follows:

$$S = \frac{VSs}{VS} \cdot 100 \quad (1)$$

where S is the solubilisation ratio expressed as a percentage, VSs is the soluble volatile solids concentration and VS refers to the total volatile solids concentration.

In order to compare the experimental data of the microalgae and WAS mixtures with the expected solubilisation ratio without substrates interaction, the theoretical solubilisation ratio was calculated using the following equation:

$$S_{calc} = f_A \cdot S_A + f_{WAS} \cdot S_{WAS} \quad (2)$$

where S_{calc} is the calculated solubilisation ratio expressed as a percentage, f_A and f_{WAS} refer to the proportion of microalgae and WAS content in each solubilisation trial, respectively, and S_A and S_{WAS} are the experimental solubilisation ratio of microalgae and WAS tested alone, respectively.

2.3.3. Microalgae and WAS co-digestion BMP assays

BMP tests were carried out in order to determine the methane yield and rate (k) of co-digestion trials with microalgae and WAS, after an autohydrolysis pretreatment. The pretreatment was applied simultaneously to both substrates, taking into account the results of the preliminary solubilisation assay in terms of exposure time (Section 2.2.2). Three conditions were tested: i) 20 % of microalgae and 80 % of WAS, ii) 50 % microalgae and 50 % of WAS and iii) 80 % of microalgae and 20% of WAS (on a VS basis). The mono-digestion of each substrate (with and without pretreatment) was also performed as control.

179 All experimental trials were prepared in triplicate with a substrate to inoculum (S/I) ratio of
180 0.5 g CODVS/g VS according to Passos et al. (2013). A blank trial without substrate was
181 used to quantify the amount of methane produced by the inoculum. After adding the proper
182 amount of both substrates and the inoculum, serum bottles (160 mL) were filled with distilled
183 water up to 100 mL, flushed with Helium gas, sealed with butyl rubber stoppers and
184 incubated at 35 ºC until biogas production ceased.
185
186 A first-order kinetic model (Equation (3)) was applied to assess the performance and the
187 kinetics of (co-)digestion assays.

$$B = B_0 \cdot [1 - \exp(-k \cdot t)] \quad (3)$$

188
189 where B represents the cumulative methane production (mL CH_4/gVS), B_0 is the final
190 methane production (mL CH_4/gVS), k refers to the first-order kinetic constant ($days^{-1}$) and t
191 is time (days).
192
193 The pair of experimental data (B,t) was adjusted by the least square method using the SOLVE
194 function from Excel. This allowed the determination of parameters k and B_0 of each co-
195 digestion assay.
196
197 Furthermore, experimental data obtained by each co-digestion mixture was compared to
198 theoretical values calculated from microalgae and WAS specific methane productions
199 (Equation (4)):

$$BMP_{calc} = f_A \cdot BMP_A + f_{WAS} \cdot BMP_{WAS} \quad (4)$$

where BMP_{calc} is the calculated BMP, f_A and f_{WAS} refer to the percentage of microalgae and WAS content in each trial, respectively, and BMP_A and BMP_{WAS} are the experimental methane yield of microalgae and WAS mono-digestions, respectively.

2.4 Analytical procedures

2.4.1 Tertiary wastewater treatment

Nutrients removal (nitrogen and phosphorous) was monitored taking samples twice per week at the end of the light phase in the photobioreactor influent (1/50 digestate/secondary effluent) and in the mixed liquor of the photobioreactor. Ortophosphate ($P\text{-}PO_4^{3-}$), nitrite ($N{-}NO_2^-$) and nitrate ($N{-}NO_3^-$) were determined using ion chromatograph DIONEX ICS1000 (Thermo-scientific, USA), operated in isocratic mode with Na_2CO_3 and $NaHCO_3$ as eluents at a temperature of 30 ºC and a flow of 1 ml/min. Values lower than 0.9 mg/L of $N{-}NO_2^-$, 1.12 of $N{-}NO_3^-$, and 0.8 mg/L of $P\text{-}PO_4^{3-}$ were considered below the limit of detection (LOD). On the other hand, ammonium ($N{-}NH_4^+$) was measured by the colorimetric method indicated in Solorzano (1969). Total inorganic nitrogen (TIN) was calculated as the sum of $N{-}NH_4^+$, $N{-}NO_2^-$ and $N{-}NO_3^-$. Samples were analyzed in triplicate. Soluble chemical oxygen demand (CODs) was determined according to Standard Methods (APHA-AWWA-WPCF, 2001).

Culture conditions as water temperature and pH were continuously measured by probes placed *in situ* and monitored by a pH-meter with a temperature sensor (Mettler Toledo, USA). Data was collected in periods of 2–3 min in a computer with the software LabVIEW®.

2.4.2 Microalgae biomass production

In order to evaluate the microalgae biomass production, turbidity was measured by means of a turbidimeter (HI 93703, HANNA Instruments, Italy) 3-5 days per week sampling at the end of the light phase. Then, total suspended solids (TSS) were determined from the correlation shown in Eq. (5) (R^2 =0.9951) between turbidity and the dry weight of algal biomass determined gravimetrically as total suspended solids according to the standard method 2540-D (APHA-AWWA-WPCF, 2001).

$$TSS\ (\frac{g}{l}) = 0.0026 \cdot Turbidity + 0.2046 \quad (5)$$

Microalgae evolution was monitored once a week using an optic microscope (Motic, China) equipped with a camera (Fi2, Nikon, Japan), connected to a computer with the software NIS-Element viewer®. Microalgae species were identified *in vivo* using conventional taxonomic books (Bourrelly, 1985; Palmer, 1962).

2.4.3 Biogas production

The total volatile solids (VS) and soluble volatile solids (VSs) were analysed according to Standard Methods (APHA AWWA-WPCF, 2001). The soluble fraction was obtained after biomass centrifugation (UNICEN20, 4200 rpm, 8min, 20 ºC) followed by filtration via glass-fiber filters (0.45 µm).

The cumulative biogas production was determined from the pressure increase in the headspace volume of the bottles measured with a manometer (GMH 3161 Greisinger,

Germany). The methane content in biogas was periodically analysed by gas chromatography, using a chromatograph with a thermal conductivity detector (Trace GC Thermo Finnigan with Hayesep packed column) and injector/detector/oven temperatures were 150, 250, 35 ºC, respectively, using helium gas as carrier.

3. Results and discussion

3.1 Wastewater treatment performance

The closed photobioreactor was operated as a tertiary wastewater treatment to remove nutrients (N and P) from the secondary effluent (treated wastewater). Additionally, it treated the digestate, which in turn increased the concentration of nutrients for microalgae growth. Although the concentration of nutrients was not constant over the experimental period, $N{-}NH_4^+$ was almost completely removed and $P\text{-}PO4^{3-}$ was never detected in the photobioreactor effluent (Figure 2). The pH was not regulated and values ranged from 9.4 to 11.5 in dark and light periods, respectively, due to the photosynthetic activity.

As shown in Figure 2, initial N-NO3- showed a decreasing pattern over time. This is due to the variations on nitrification processes in the secondary effluent caused by seasonal changes in the HRAP performance (Arias et al., 2017 and Garcia et al., 2000), leading to changes in N-NO3- concentrations in the influent. In any way, the average removal during the period of the experiment was 58 %. Indeed, the lack of $N{-}NH_4^+$ could enhance nitrates consumption as nitrogen source by microalgae since it has been shown that microalgae tend to prefer $N{-}NH_4^+$ over $N\text{-}NO_3^-$, and nitrate consumption does not occur until $N{-}NH_4^+$ is almost completely consumed (Garcia et al., 2000).

Regarding the CODs, the average concentration in the influent was 141±4 mg/L, which was reduced by 50.6 % over the first 3 weeks of operation and 70 % during the last 2 weeks (Figure 2). This increase in the COD removal efficiency during the last 2 weeks might be caused by an increment in the proportion of biodegradable organic matter in the influent. Notwithstanding, the CODs of the photobioreactor effluent was always below the discharge limit of 125 mg O_2/L (Directive 98/15/EC, 1998).

The biomass was clearly dominated by *Scenedesmus* sp. In general, the performance of this culture as a tertiary treatment for the digestate diluted in secondary effluent is comparable to other studies using different microalgae species that typically grow on wastewater. Olguín et al. (2003) treated anaerobically digested pig slurries diluted in seawater, and achieved removals around 90, 87 and 50 % for $N{-}NH_4^+$, $P\text{-}PO4^{3-}$ and COD, respectively. Similar results were obtained by Cañizares et al. (1994), achieving removals above 90 % in both $N{-}NH_4^+$ and $P\text{-}PO4^{3-}$ during the treatment of the pretreated pig slurries with *Spirulina maxima*.

In previous studies most of the removal efficiencies achieved with different microalgae consortia range between 60 % and 99 % (Olguín et al., 2003; Ruiz-Marin et al., 2010; Van Den Hende et al., 2016; Viruela et al., 2016). Such removals demonstrate that in general, algae-based wastewater treatment systems are a feasible alternative for nutrients and organic matter removal regardless of the type of culture. Remarkably, the results of this study reached higher removals of NH_4^+ and $P\text{-}PO_4^{3-}$ in comparison to the study of Viruela et al. (2016) and Wang et al. (2010) treating only anaerobic effluents (centrate), and the study of Arias et al. (2017), treating microalgae digestate diluted with secondary effluents. This fact could be

294 directly influenced by an efficient uptake of nutrients by microalgae, which can be considered
295 by means of the high biomass concentration reached in this study. Additionally, the HRT of
296 8 d might be also contributing to the high removals obtained in both nutrients and COD in
297 this study. Indeed long retention times are recommended to improve removal efficiencies in
298 cases of low nutrients availability (Munoz and Guieysse, 2006).
299
300 ***3.2 Microalgae growth***
301 As shown in Table 1, the secondary effluent had low $N{-}NH_4^+$ concentration (0.5 mg/L) and
302 the digestate provided an additional $N{-}NH_4^+$ and phosphorous source to the photobioreactor
303 which enhanced microalgae growth. During the experiment, the biomass showed an
304 exponential growth during the first 5 days, increasing the initial concentration of 0.5 gTSS/L
305 by 57.0 %. After that, a constant concentration of 1.1±0.1 gTSS/L was achieved and
306 maintained throughout the experiment. The high biomass obtained in this study suggests the
307 utilization of all the influent dissolved inorganic N and P available in form of N−NH4+,
308 N−NO3- and P−PO43-, but also of other organic forms of N and P as shown by (García et
309 al., 2002).
310
311 At the beginning the mixed culture was mainly dominated by *Stigeoclonium* sp. However,
312 after the 10th day, the culture was clearly dominated by *Scenedesmus* sp. This could be
313 influenced by the N/P ratio (12:1) in the photobioreactor. Indeed, Viruela et al., (2016) and
314 Xin et al., (2010) reported ratios from 5:1 to 12:1 to be the optimal for the dominance of
315 *Scenedesmus* sp. over other species. This specie in particular is known to have high growth
316 rate in spite of low nutrients availability, specially to P limitation (Cai et al., 2013; Xin et al.,
317 2010). In addition to nutrients availability in the culture, high adaptability of this genus to

several factors could facilitate their dominance over other green microalgae and cyanobacteria. These factors include high tolerance to light limitation (Liu et al., 2017) as well as high light intensities (Huisman et al., 1999), efficient adaptation to wide ranges of pH from 7.1 (Zhang et al., 2014) to 10.5 (da Fontoura et al., 2015). Indeed, their adaptability to grow in the digestate of different biomass feedstocks has already been demonstrated (Marcilhac et al., 2014; Uggetti et al., 2014a). These studies highlighted their capacity to grow under high $N{-}NH_4^+$ content, phosphorous limitation and high pH. Furthermore, this species is among the fastest growing green microalgae in wastewater and produce high yields in terms of carbohydrates or lipids (Komolafe et al., 2014; Rodolfi et al., 2009), which represents an advantage in terms of their conversion to biogas or biofuels.

In addition to *Scenedesmus* sp., a variety of microalgae and cyanobacteria have shown the capacity to grow on diluted and undiluted digestates from various sources. For instance, the digestate from swine slurry (Cheng et al., 2015), sewage sludge (Uggetti et al., 2014b), abattoir digestate (Bchir et al., 2011), swine manure (Hu et al., 2012) and poultry manure (Iyovo et al., 2010) are adequate for microalgae biomass production. Regarding the studies focused on recycling microalgae digestate for biomass production, Prajapati et al., (2014) used the digestate from anaerobic digestion of *Chroococcus* sp. diluted in tap water as nutrient supplement for microalgal growth. In that case, the microalgae concentration was 0.8±0.1g TSS/L in a batch process. Likewise, in the study of Arias et al. (2017), digestate diluted with secondary effluent was employed to grow and select cyanobacteria, achieving a biomass production between 0.4 and 1.05 g TSS/L. In our research, higher concentrations (1.1±0.1 g TSS/L) were reached by utilizing digestate diluted with secondary effluent under semi-continuous mode.

342

343 ***3.3 Autohydrolysis pretreatment effect on biomass solubilisation***

344 The effect of the autohydrolysis pretreatment was initially evaluated by the biomass
345 solubilisation increase (Figure 3). WAS reached the highest solubilisation ratio (25.7 %) and
346 microalgae the lowest (11.4 %). In view of the results, microalgae showed to be less
347 biodegradable than WAS due to the resistant structure of their cell wall. case in particular,
348 *Scenedesmus* has been reported to have a complex multilayer cell wall (Tukaj and
349 Bohdanowicz, 1995).

350

351 The results obtained in this study are in accordance with those obtained by Mahdy et al.,
352 (2015), who observed higher solubilisation rates with WAS than microalgae after a thermal
353 pretreatment at 120 ºC for 40 min. Besides, similar solubilisation rates for WAS were
354 obtained by Carvajal et al. (2013) (25 % for proteins and 21 % for carbohydrates), who
355 studied how inherent enzymes of WAS were released by applying a thermal pretreatment at
356 55 ºC.

357

358 Considering the mixed substrates, at the end of the assay the solubilisation ratios were 21 %
359 and 15 % for the mixtures with 50 % and 80 % of microalgae, respectively. Indeed, the
360 solubilisation ratio decreased proportionally to the concentration of WAS decrease
361 (R^2=0.95). This proportionality was confirmed by comparing experimental data with
362 theoretical solubilisation ratios, calculated from Equation (2). This means that there was no
363 co-pretreatment effect, since microalgae solubilisation was not improved by pretreating it
364 together with WAS. Therefore, inherent enzymes of WAS released during the autohydrolysis
365 pretreatment were not effective at disrupting microalgae cell wall.

366

367 Finally, Figure 3 shows that all assays reached an asymptote by the end of the assay, meaning
368 that solubilisation ratio increase was stabilised by that time. An increase on the contact time
369 would not entail a significant increase of substrate solubilisation, whereas it would increase
370 the amount of energy needed for the pretreatment. Therefore, 7.5 hours was selected as the
371 optimum contact time for the autohydrolysis pretreatment prior to biochemical methane
372 potential assays. This is in accordance with our previous studies which showed that a contact
373 time of 8 hours was the optimum when pretreating microalgae at low temperature (Passos et
374 al., 2013).

375

376 ***3.4 Biochemical methane potential of pretreated microalgae and WAS co-digestion***

377 The anaerobic co-digestion BMP assays lasted 41 days (Figure 4). Regarding the pure
378 substrates, WAS showed the highest methane yield (139 mL CH_4/g VS) while microalgae
379 presented the lowest (82 mL CH_4/g VS) (Table 2). Nonetheless, after the pretreatment,
380 microalgae presented a higher increase with respect to WAS. Indeed, the pretreatment
381 applied to microalgae increased the methane yield by 64 %, achieving a value of 134 mL
382 CH_4/g VS. On the other hand, pretreated WAS showed a production of 204 mL CH_4/g VS,
383 which represents an increase of 47 %. These results are in accordance with the literature
384 highlighting the importance of microalgae pretreatment, since their resistant cell wall
385 hampers microalgae hydrolysis and anaerobic fermentation (Passos et al., 2014). Particularly,
386 *Scenedesmus* sp. has a complex rigid cell wall which makes even more difficult the
387 accessibility of enzymes to the substrate during the digestion process (González-Fernández
388 et al., 2012).

389

390 The cumulative methane yield of the co-digestion trials were 187 mL CH_4/g VS, 162 mL
391 CH_4/g VS and 132 mL CH_4/g VS for the mixtures of WAS with 20 %, 50 % and 80 % of
392 microalgae, respectively. In order to detect potential co-digestion synergies, the theoretical
393 methane yields were calculated according to Equation (4). The results showed neither
394 positive nor negative synergies between substrates, meaning that the co-digestion did not
395 improve microalgae anaerobic biodegradability. The lack of WAS enzymes effect on
396 *Scenedesmus* sp. cell wall disruption, or the low C/N ratio might be responsible for the lack
397 of synergies. These results are in agreement with Costa et al. (2012), who studied the co-
398 digestion of macroalgae species (*Ulva* and *Gracilaria*) with WAS without any pretreatment.
399 Additionally, Neumann et al. (2015) studied the co-digestion of *Botryococcus braunii* and
400 WAS and synergies were neither identified. On the contrary, Wang et al. (2013) observed 23
401 % increase in biogas production when co-digesting *Chorella* sp. and WAS, with 41 % of
402 microalgae. Despite *Chorella* sp. has a rigid cell wall due to its high content of cellulose, the
403 co-digestion with WAS enhanced the hydrolysis.
404
405 The methane content in biogas of each co-digestion assay was periodically measured (Table
406 2). Results showed no differences among trials. Thus, the methane content was independent
407 of the ratio between co-digestion substrates (Caporgno et al., 2015) and it was neither
408 affected by the autohydrolysis pretreatment nor by the co-digestion.
409
410 Moreover, the methane production rate was also analysed through the apparent kinetic
411 constant (k) of the first-order experimental model, as defined in Equation (3). Table 2 shows
412 that substrates without pretreatment had the lowest values of k (0.16 $days^{-1}$ and 0.17 $days^{-1}$
413 for microalgae and WAS, respectively), whereas pretreated substrates increased their kinetic

414 constants up to 0.27 $days^{-1}$ and 0.25 day^{-1} for microalgae and WAS, respectively. Thus, a
415 significant increase of the production rate (69 % for microalgae and 47 % for WAS) was
416 observed by applying the pretreatment. Moreover, the co-digestion trials showed higher
417 kinetic constants (0.29 $days^{-1}$, 0.32 $days^{-1}$ and 0.30 $days^{-1}$ for 20 %, 50 % and 80 % of
418 microalgae content co-digestions) as compared to the mono-digestions. This evidenced how
419 the co-digestion of microalgae and WAS can improve the mono-digestion of both substrates.
420 Costa et al. (2012), Neumann et al. (2015) and Wang et al. (2013) agreed that co-digestion
421 of microalgae and WAS improved the kinetic constant despite having different conclusion in
422 terms of the final methane yield. This result was considered the main advantage of the studied
423 microalgae and WAS co-digestion, as it may reduce the time needed for reaching the highest
424 biogas production. This means that lower hydraulic retention times, hence smaller digesters
425 could be used, reducing the costs.
426
427 ***3.5 The approach of recycling nutrients in a bioenergy producing system***
428
429 This study highlights the viability of integrating an algae-based tertiary wastewaster
430 treatment system in a conventional WWTP that includes both processes: activated sludge and
431 anaerobic digestion. This short term study also offers an alternative to the recycling use of
432 digestate.

433 Although the reuse of digestate as biofertilizer can promote a sustainable biogas production
434 (Solé-Bundó et al., 2017), this substrate can be combined with secondary effluents as an
435 alternative substrate to produce microalgal biomass. Additionally, this process could improve
436 the treatment of remaining nutrients from secondary effluents and taking advantage of the

nutrients contained in the digestate. Considering the promising results here included, further studies based in long term conditions are recommended. This approach would involve a promising opportunity to close the biorefinery loop, accomplishing a sustainable and self-supporting use of resources and reducing disposal costs and environmental impacts.

4. Conclusions

Microalgal anaerobic digestate diluted with secondary wastewater was an effective source of nitrogen and phosphorus for microalgae growth in a photobioreactor. A complete uptake of $N\text{-}NH_4^+$ and $P\text{-}PO_4^{3-}$ was observed, while a constant production of 1.1 gTSS/L of algal biomass was achieved. This biomass, mainly composed by *Scenedesmus* sp., supported a low methane yield (82 $mlCH_4/gVS$) that was improved by 130 % after an autohydrolysis co-pretreatment and co-digestion with waste activated sludge. Thus, integrating microalgae tertiary treatment into activated sludge systems is a promising and feasible solution to recover energy and nutrients from waste, improving wastewater treatment plants sustainability.

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References

1. APHA-AWWA-WPCF, 2001. Standard methods for the examination of water and waste water, 20th ed. America Public Health Association, Washington DC.
2. Arias, D.M., Uggetti, E., García-Galán, M.J., García, J., 2017. Cultivation and selection of cyanobacteria in a closed photobioreactor used for secondary effluent and digestate treatment. Sci. Total Environ. 587–588, 157–167. doi:10.1016/j.scitotenv.2017.02.097
3. Bchir, F.S., Gannoun, H., El Herry, S., Hamdi, M., 2011. Optimization of Spongiochloris sp. biomass production in the abattoir digestate. Bioresour. Technol. 102, 3869–3876. doi:10.1016/j.biortech.2010.11.036
4. Beltran, C., Jeison, D., Fermoso, F.G., Borja, R., 2016. Batch anaerobic co-digestion of waste activated sludge and microalgae (Chlorella sorokiniana) at mesophilic temperature. J. Environ. Sci. Health. A. Tox. Hazard. Subst. Environ. Eng. 51, 847–850. doi:10.1080/10934529.2016.1181456
5. Bourrelly, P., 1985. Les algues d'eau douce, in: Les Algues Vertes. Societé nouvelle des éditions doubée.
6. Cai, T., Park, S.Y., Li, Y., 2013. Nutrient recovery from wastewater streams by microalgae: Status and prospects. Renew. Sustain. Energy Rev. 19, 360–369. doi:10.1016/j.rser.2012.11.030
7. Cañizares, R.O., Rivas, L., Montes, C., Domínguez, a. R., Travieso, L., Benitez, F.,

1994. Aerated swine-wastewater treatment with K-carrageenan-immobilized Spirulina maxima. Bioresour. Technol. 47, 89–91. doi:10.1016/0960-8524(94)90035-3

8. Caporgno, M.P., Trobajo, R., Caiola, N., Ibáñez, C., Fabregat, A., Bengoa, C., 2015. Biogas production from sewage sludge and microalgae co-digestion under mesophilic and thermophilic conditions. Renew. Energy 75, 374–380. doi:10.1016/j.renene.2014.10.019
9. Carvajal, A., Peña, M., Pérez-Elvira, S., 2013. Autohydrolysis pretreatment of secondary sludge for anaerobic digestion. Biochem. Eng. J. 75, 21–31. doi:10.1016/j.bej.2013.03.002
10. Cheng, J., Xu, J., Huang, Y., Li, Y., Zhou, J., Cen, K., 2015. Growth optimisation of microalga mutant at high CO2 concentration to purify undiluted anaerobic digestion effluent of swine manure. Bioresour. Technol. 177, 240–246. doi:10.1016/j.biortech.2014.11.099
11. Chisti, Y., 2008. Biodiesel from microalgae beats bioethanol. Trends Biotechnol. 26, 126–131. doi:10.1016/j.tibtech.2007.12.002
12. Costa, J.C., Gonçalves, P.R., Nobre, A., Alves, M.M., 2012. Biomethanation potential of macroalgae Ulva spp. and Gracilaria spp. and in co-digestion with waste activated sludge. Bioresour. Technol. 114, 320–326. doi:10.1016/j.biortech.2012.03.011
13. da Fontoura, J.T., Rotermund, S., Araujo, A.L., Ramirez, N., Rubleske, M., Farenzena, M., Gutterres, M., 2015. Tannery Wastewater Treatment with Scenedesmus sp . 1–10.
14. Directive 98/15/EC, 1998. 98/15/EC of 27 February. Off. J. Eur. Communities.

doi:http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex:31998L0015

15. García, J., Hernández-Mariné, M., Mujeriego, R., 2002. Analysis of key variables controlling phosphorus removal in high rate oxidation ponds provided with clarifiers. Water SA 28, 55–62.
16. Garcia, J., Mujeriego, R., Hernandez-Marine, M., 2000. High rate algal pond operating strategies for urban wastewater nitrogen removal. Appl. Phycol. 12, 331–339. doi:10.1023/a:1008146421368
17. González-Fernández, C., Sialve, B., Bernet, N., Steyer, J.P., 2012. Thermal pretreatment to improve methane production of Scenedesmus biomass. Biomass and Bioenergy 40, 105–111. doi:10.1016/j.biombioe.2012.02.008
18. Gutiérrez, R., Ferrer, I., Uggetti, E., Arnabat, C., Salvadó, H., García, J., 2016. Settling velocity distribution of microalgal biomass from urban wastewater treatment high rate algal ponds. Algal Res. 16, 409–417. doi:10.1016/j.algal.2016.03.037
19. Hu, B., Min, M., Zhou, W., Du, Z., Mohr, M., Chen, P., Zhu, J., Cheng, Y., Liu, Y., Ruan, R., 2012. Enhanced mixotrophic growth of microalga Chlorella sp. on pretreated swine manure for simultaneous biofuel feedstock production and nutrient removal. Bioresour. Technol. 126, 71–79. doi:10.1016/j.biortech.2012.09.031
20. Huisman, J., Joanker, R.R., Zonneveld, C., Weissing, F.J., 1999. Competition for light between phytoplankton species: experimental tests of mechanistic theory. Ecology 80, 211–222.
21. Iyovo, G.D., Du, G., Chen, J., 2010. Sustainable Bioenergy Bioprocessing: Biomethane Production, Digestate as Biofertilizer and as Supplemental Feed in Algae Cultivation to Promote Algae Biofuel Commercialization. J. Microb.

530 Biochem. Technol. 2, 100–106. doi:10.4172/1948-5948.1000032
531 22. Komolafe, O., Velasquez Orta, S.B., Monje-Ramirez, I., Noguez, I.Y., Harvey,
532 A.P., Orta Ledesma, M.T., 2014. Biodiesel production from indigenous microalgae
533 grown in wastewater. Bioresour. Technol. 154, 297–304.
534 doi:10.1016/j.biortech.2013.12.048
535 23. Liu, L., Fan, H., Liu, Y., Liu, C., Huang, X., 2017. Bioresource Technology
536 Development of algae-bacteria granular consortia in photo-sequencing batch
537 reactor. Bioresour. Technol. 232, 64–71. doi:10.1016/j.biortech.2017.02.025
538 24. Mahdy, A., Mendez, L., Ballesteros, M., González-Fernández, C., 2015.
539 Algaculture integration in conventional wastewater treatment plants: Anaerobic
540 digestion comparison of primary and secondary sludge with microalgae biomass.
541 Bioresour. Technol. 184, 236–244. doi:10.1016/j.biortech.2014.09.145
542 25. Marcilhac, C., Sialve, B., Pourcher, A.M., Ziebal, C., Bernet, N., Béline, F., 2014.
543 Digestate color and light intensity affect nutrient removal and competition
544 phenomena in a microalgal-bacterial ecosystem. Water Res. 64, 278–287.
545 doi:10.1016/j.watres.2014.07.012
546 26. Muga, H.E., Mihelcic, J.R., 2008. Sustainability of wastewater treatment
547 technologies. J. Environ. Manage. 88, 437–47. doi:10.1016/j.jenvman.2007.03.008
548 27. Munoz, R., Guieysse, B., 2006. Algal–bacterial processes for the treatment of
549 hazardous contaminants: a review. Water Res. 40, 2799–2815.
550 28. Neumann, P., Torres, A., Fermoso, F.G., Borja, R., Jeison, D., 2015. Anaerobic co-
551 digestion of lipid-spent microalgae with waste activated sludge and glycerol in
552 batch mode, International Biodeterioration & Biodegradation.
553 doi:10.1016/j.ibiod.2015.01.020

554 29. Olguín, E.J., Galicia, S., Mercado, G., Pérez, T., 2003. Annual productivity of
555 Spirulina (Arthrospira) and nutrient removal in a pig wastewater recycling process
556 under tropical conditions. J. Appl. Phycol. 15, 249–257.
557 doi:10.1023/A:1023856702544
558 30. Palmer, C.M., 1962. Algas en los abastecimientos de agua. Manual ilustrado acerca
559 de la identificación, importancia y control de las algas en los abastecimientos de
560 agua. Editorial Interamericana, México.
561 31. Passos, F., Ferrer, I., 2014. Microalgae conversion to biogas: Thermal pretreatment
562 contribution on net energy production. Environ. Sci. Technol. 48, 7171–7178.
563 doi:10.1021/es500982v
564 32. Passos, F., García, J., Ferrer, I., 2013. Impact of low temperature pretreatment on
565 the anaerobic digestion of microalgal biomass. Bioresour. Technol. 138, 79–86.
566 doi:10.1016/j.biortech.2013.03.114
567 33. Passos, F., Gutiérrez, R., Brockmann, D., Steyer, J.-P., García, J., Ferrer, I., 2015.
568 Microalgae production in wastewater treatment systems, anaerobic digestion and
569 modelling using ADM1. Algal Res. 10, 55–63. doi:10.1016/j.algal.2015.04.008
570 34. Passos, F., Uggetti, E., Carrère, H., Ferrer, I., 2014. Pretreatment of microalgae to
571 improve biogas production: A review. Bioresour. Technol. 172, 403–412.
572 doi:10.1016/j.biortech.2014.08.114
573 35. Pittman, J.K., Dean, A.P., Osundeko, O., 2011. The potential of sustainable algal
574 biofuel production using wastewater resources. Bioresour. Technol. 102, 17–25.
575 doi:10.1016/j.biortech.2010.06.035
576 36. Prajapati, S.K., Kumar, P., Malik, A., Vijay, V.K., 2014. Bioconversion of algae to
577 methane and subsequent utilization of digestate for algae cultivation: A closed loop

578 bioenergy generation process. Bioresour. Technol. 158, 174–180.
579 doi:10.1016/j.biortech.2014.02.023
580 37. Rodolfi, L., Zittelli, G.C., Bassi, N., Padovani, G., Biondi, N., Bonini, G., Tredici,
581 M.R., 2009. Microalgae for oil: strain selection, induction of lipid synthesis and
582 outdoor mass cultivation in a low-cost photobioreactor 102, 100–112.
583 doi:10.1002/bit.22033
584 38. Ruiz-Marin, A., Mendoza-Espinosa, L.G., Stephenson, T., 2010. Growth and
585 nutrient removal in free and immobilized green algae in batch and semi-continuous
586 cultures treating real wastewater. Bioresour. Technol. 101, 58–64.
587 doi:10.1016/j.biortech.2009.02.076
588 39. Solé-Bundó, M., Cucina, M., Folch, M., Tàpias, J., Gigliotti, G., Garfí, M., Ferrer,
589 I., 2017. Assessing the agricultural reuse of the digestate from microalgae anaerobic
590 digestion and co-digestion with sewage sludge. Sci. Total Environ. 586, 1–9.
591 doi:10.1016/j.scitotenv.2017.02.006
592 40. Solorzano, L., 1969. Determination of ammonia in natural waters by the
593 phenolhypochlorite method. Limnol. Ocean. 14, 799.
594 41. Tukaj, Z., Bohdanowicz, J., 1995. Sensitivity to fuel oil and cell wall structure of
595 some Scenedesmus (Chlorococcales) strains. Acta Soc. Bot. Pol. 64, 139–147.
596 42. Uggetti, E., Sialve, B., Latrille, E., Steyer, J.P., 2014a. Anaerobic digestate as
597 substrate for microalgae culture: The role of ammonium concentration on the
598 microalgae productivity. Bioresour. Technol. 152, 437–443.
599 doi:10.1016/j.biortech.2013.11.036
600 43. Uggetti, E., Sialve, B., Trably, E., Steyer, J.-P., 2014b. Integrating microalgae
601 production with anaerobic digestion: a biorefinery approach. Biofuels, Bioprod.

602 Biorefining 8, 516–529. doi:10.1002/bbb.1469
603 44. Van Den Hende, S., Beelen, V., Julien, L., Lefoulon, A., Vanhoucke, T., Coolsaet,
604 C., Sonnenholzner, S., Vervaeren, H., Rousseau, D.P.L., 2016. Technical potential
605 of microalgal bacterial floc raceway ponds treating food-industry effluents while
606 producing microalgal bacterial biomass : An outdoor pilot-scale study. Bioresour.
607 Technol. 218, 969–979. doi:10.1016/j.biortech.2016.07.065
608 45. Viruela, A., Murgui, M., Gómez-gil, T., Durán, F., Robles, Á., Victoria, M., Ferrer,
609 J., Seco, A., 2016. Water resource recovery by means of microalgae cultivation in
610 outdoor photobioreactors using the effluent from an anaerobic membrane bioreactor
611 fed with pre-treated sewage. Bioresour. Technol. 218, 447–454.
612 doi:10.1016/j.biortech.2016.06.116
613 46. Wang, L., Min, M., Li, Y., Chen, P., Chen, Y., Liu, Y., Wang, Y., Ruan, R., 2010.
614 Cultivation of green algae Chlorella sp. indifferent wastewaters from municipal
615 wastewater treatment plant. Appl. Biochem. Biotechnol. 162, 1174–1186.
616 47. Wang, M., Sahu, A.K., Rusten, B., Park, C., 2013. Anaerobic co-digestion of
617 microalgae Chlorella sp. and waste activated sludge. Bioresour. Technol. 142, 585–
618 590. doi:10.1016/j.biortech.2013.05.096
619 48. Xin, L., Hong-ying, H., Ke, G., Ying-xue, S., 2010. Effects of different nitrogen and
620 phosphorus concentrations on the growth , nutrient uptake , and lipid accumulation
621 of a freshwater microalga Scenedesmus sp . Bioresour. Technol. 101, 5494–5500.
622 doi:10.1016/j.biortech.2010.02.016
623 49. Zhang, T.-Y., Wu, Y.-H., Hu, H.-Y., 2014. Domestic wastewater treatment and
624 biofuel production by using microalga Scenedesmus sp . Water Sci. Technol. 69,
625 2492–2497. doi:10.2166/wst.2014.160

626 50. Zhen, G., Lu, X., Kobayashi, T., Kumar, G., Xu, K., 2016. Anaerobic co-digestion
627 on improving methane production from mixed microalgae (Scenedesmus sp .,
628 Chlorella sp .) and food waste : kinetic modelling and synergistic impact evaluation.
629 Chem. Eng. J. doi:10.1016/j.cej.2016.04.118
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631

632 **Table 1**. Composition of the wastewater used as photobioreactor feedstock.

Parameter	Digestate	Secondary effluent	Photobioreactor influent[a]
pH	-	-	7.9 ± 0.3
TSS (g/L)	13.4 ± 8.5	[b]	0.26 ± 0.17
VSS (g/L)	12.3 ± 6.5	[b]	0.24 ± 0.13
Alkalinity (mg $CaCO_3$/L)	-	-	153 ± 38.4
CODs (mg O_2/L)	122.8 ± 25.9	18.3 ± 5.5	141.1 ± 36.1
$N{-}NH_4^+$ (mg/L)	459 ± 166.5	0.21 ± 0.84	9.17 ± 3.33
$N\text{-}NO_2^-$ (mg/L)	<LOD[c]	1.44 ± 0.69	1.53 ± 0.91
$N\text{-}NO_3^-$ (mg/L)	<LOD[c]	15.94 ± 4.94	15.94 ± 4.94
TIN	-	-	26.64 ± 3.06
$P\text{-}PO_4^{3-}$ (mg/L)	<LOD[c]	2.18 ± 0.87	2.18 ± 0.87

TIN: Total Inorganic Nitrogen

[a]*Photobioreactor influent prepared by diluting the digestate in secondary effluent (1:50 ratio).*

[b]*TSS and VSS in the secondary effluent presented values <0.03 g* L^{-1}.

[c] *LOD: Limit of Detection.*

633

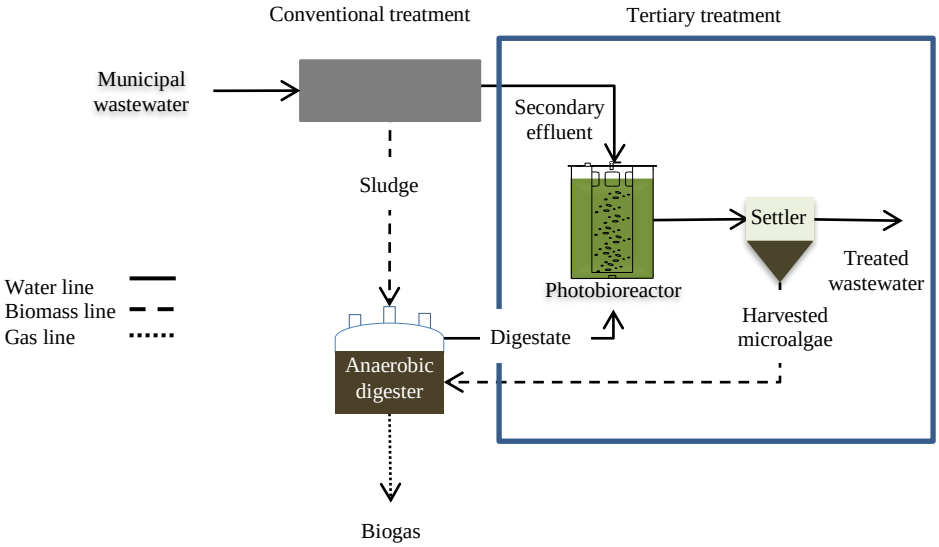
634 **Table 2.** Experimental results and data analysis at the end of the biochemical methane

635 potential assays.

	Methane yield	% CH_4	***k***
	mg CH_4/g VS	%	day^{-1}
Microalgae (M)	82 ± 10	63.3 ± 0.1	0.16
WAS	139 ± 3	63.9 ± 0.8	0.17
(M)p	134 ± 6	64.0 ± 0.1	0.27
(WAS)p	204 ± 3	63.5 ± 0.3	0.25
(20 %M+80 %WAS)p	187 ± 9	64.0 ± 0.4	0.29
(50 %M+50 %WAS)p	162 ± 6	64.3 ± 0.9	0.32
(80 %M+20 %WAS)p	132 ± 2	64.6 ± 0.7	0.30

p = pretreated

636



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639 **Figure 1.** General scheme of the system proposed in this study.

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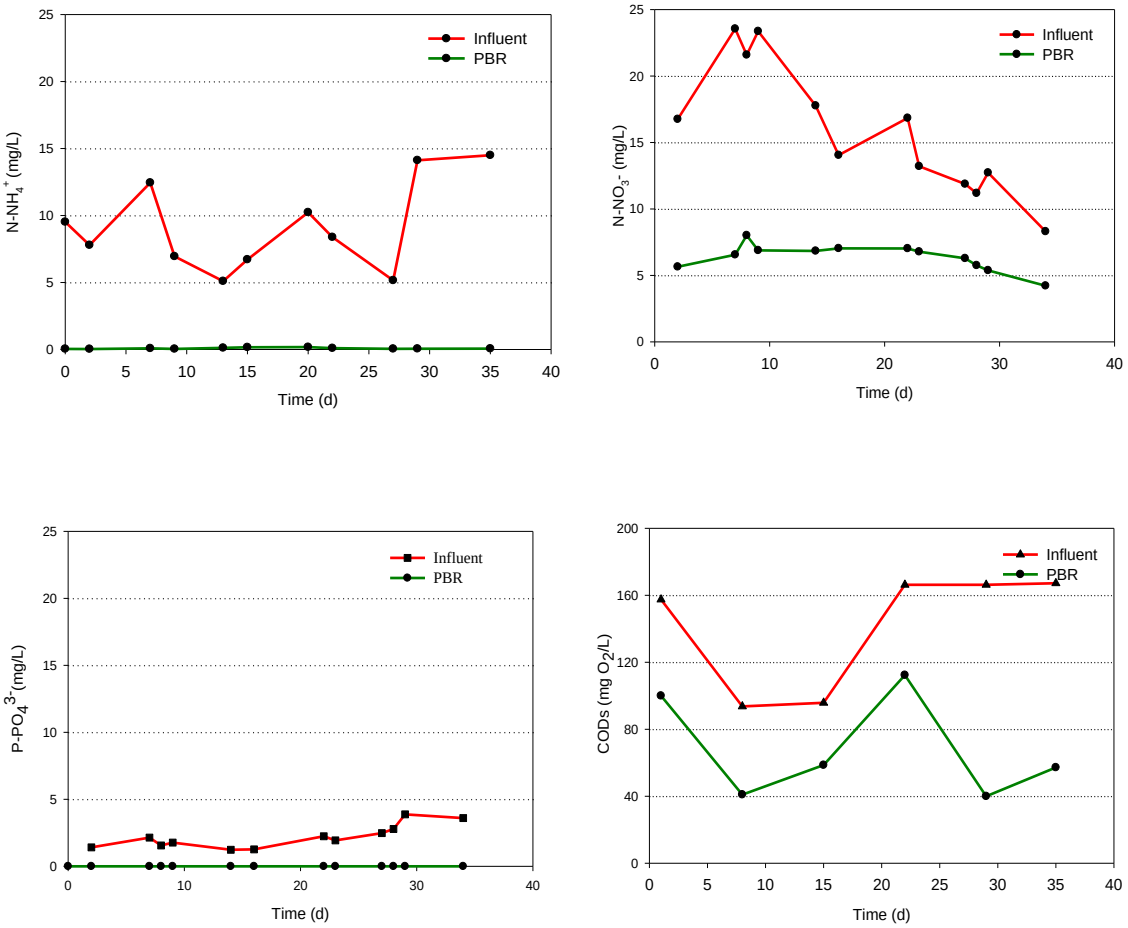

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643

644 **Figure 2.** Influent and photobioreactor concentrations of ammonium ($N{-}NH_4^+$),

645 ortophosphates ($P{-}PO_4^{3-}$), nitrates ($N{-}NO_3^-$) and soluble chemical oxygen demand (CODs).

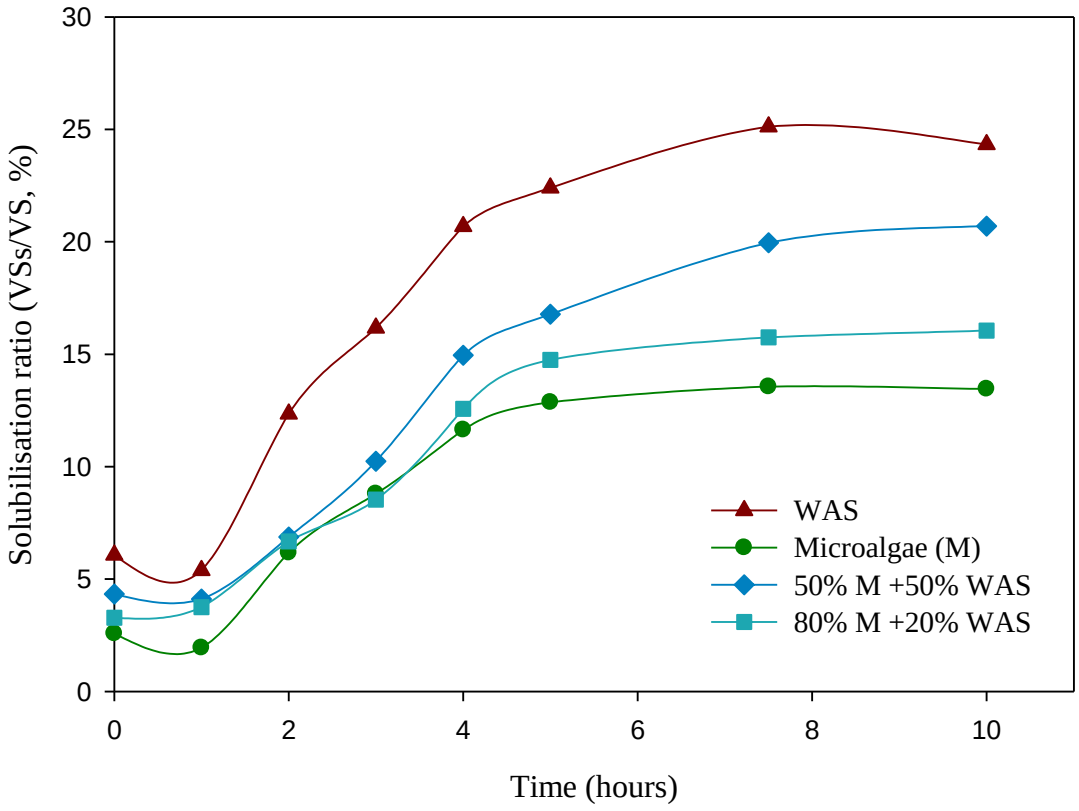

Figure 3. Solubilisation ratio over the solubilisation assay (10 h).

Note: M= microalgae; WAS= waste activated sludge.

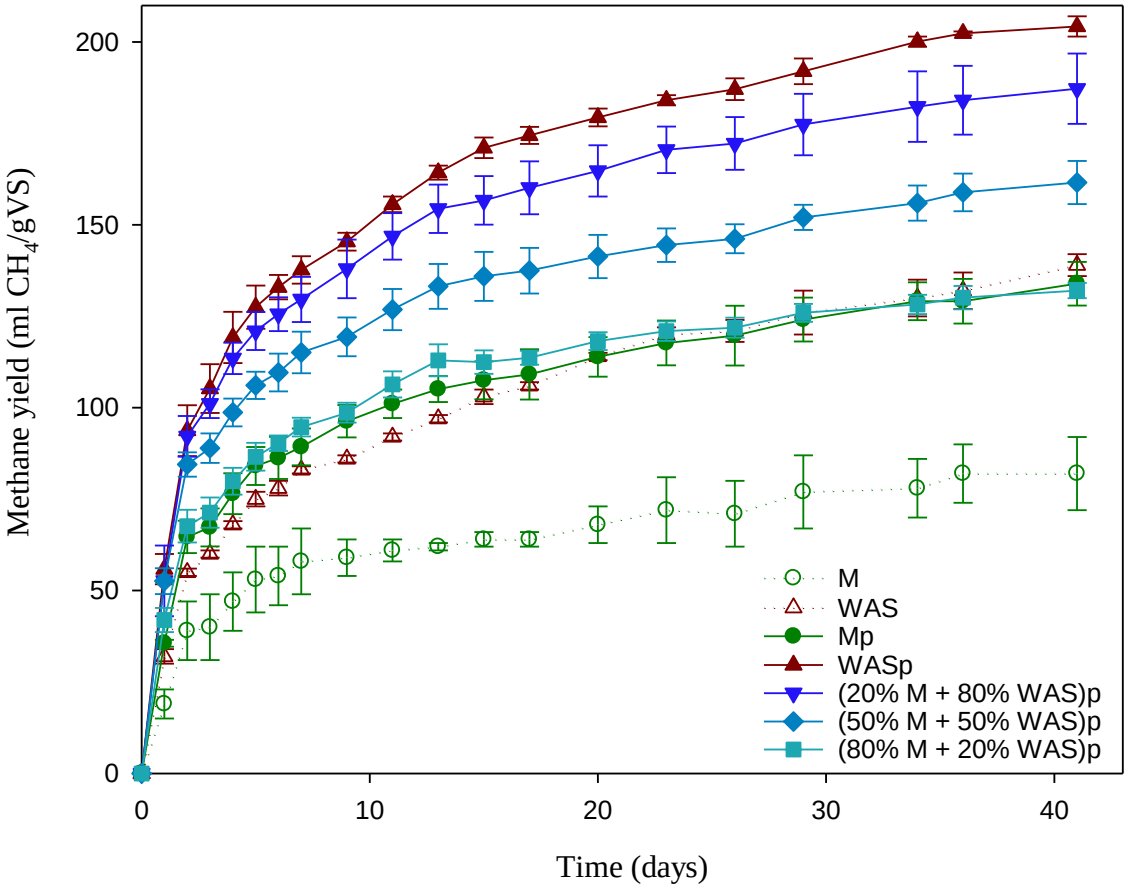

Figure 4. Cumulative methane yield (mg CH_4/g VS) over the biochemical methane potential assays with *Scenesdesmus* sp. and WAS (co-digestion and mono-digestion). Symbols represent the mean value and standard deviation.

Note: M= microalgae; WAS= waste activated sludge; p = pretreated