Photosynthetic biogas upgrading to bio-methane: boosting nutrient recovery via biomass productivity control.

Alma Toledo-Cervantes[1], Mayara L. Serejo[2], Saúl Blanco[3], Rebeca Pérez[1], Raquel Lebrero[1], Raúl Muñoz[1]*

1- Department of Chemical Engineering and Environmental Technology, University of Valladolid, Dr. Mergelina s/n., Valladolid 47011, Spain.

2- Faculty of Engineering, Architecture and Urbanism and Geography, Federal University of Mato Grosso do Sul, Campo Grande 79090-900, Brazil.

3- Department of Biodiversity and Environmental Management, University of León, León 24071, Spain. Current address: The Institute of the Environment, La Serna, 58, 24007 León, Spain.

*Corresponding author: mutora@iq.uva.es

Abstract.

A pilot high rate algal pond (HRAP) interconnected to an external CO_2−H_2S absorption column via settled broth recirculation was used to simultaneously treat a synthetic digestate and to upgrade biogas to a bio-methane with sufficient quality to be injected into natural gas grids. An innovative HRAP operational strategy with biomass recirculation based on the control of algal-bacterial biomass productivity (2.2, 4.4 and 7.5 g m^{-2} d^{-1}) via settled biomass wastage was evaluated in order to enhance nutrient recovery from digestate at a constant hydraulic retention time. The influence of the recycling liquid to biogas (L/G) ratio on the quality of the upgraded biogas was assessed. The bio-methane composition under a L/G ratio of 1 (0.4 ± 0.1% CO_2, 0.03 ± 0.04% O_2, 2.4 ± 0.2% N_2 and 97.2 ± 0.2% CH_4) complied with the technical specifications of most European bio-methane legislations regardless of the biomass productivity established. The HRAP operational strategy applied allowed increasing the N and P recovery from 19 and 22% to 83 and 100%, respectively, when the biomass productivity was increased from 2.2 to 7.5 g m^{-2} d^{-1}. Finally, the dynamics of microalgae and bacteria population structure were characterized by morphological identification and DGGE analysis.

Keywords: Biogas upgrading; bio-methane; microalgae-based processes; nutrients recovery; wastewater treatment.

Highlights:

- A removal of CO_2 and H_2S from biogas higher than 99% was achieved.
- A low L/G ratio prevented O_2 and N_2 contamination of the upgraded biogas.
- The bio-methane complied with EU legislation for injection into natural gas grids.
- A novel HRAP operation based on biomass productivity control was developed.
- This operation strategy allowed maximizing nutrient recovery from digestate.

Introduction.

Anaerobic digestion offers a cost-effective and environmentally feasible solution for organic waste management while contributing to satisfy the global demand for renewable energy via biogas production. In this context, the annual biogas production in the European Union accounted for ~13.4 Mtoe in 2013 [1]. Biogas is composed mainly of methane (CH_4) (40-75%), carbon dioxide (CO_2) (25-50%), hydrogen sulfide (H_2S) (0.005-2%) and ammonia (NH_3) (<1%). Other gases such as hydrogen (H_2), nitrogen (N_2), oxygen (O_2) and halogenated hydrocarbons are also present in raw biogas at lower concentrations [2]. The concentration of these biogas pollutants depends on the composition of the initial organic substrate and the type of anaerobic digestion process. The H_2S present in biogas corrodes metal parts, reduces the durability of the motors and generates hazardous sulfur dioxide when biogas is combusted for the generation of heat and electricity. Likewise, CO_2 reduces the specific calorific value of biogas and increases carbon monoxide and hydrocarbon emissions during combustion. Therefore, these biogas pollutants must be previously removed in order to comply with the technical specifications for biogas to be used as a transport fuel or injected into natural gas grids. Most international legislations for bio-methane, which is the most common term to refer to the upgraded biogas, require concentrations of CH_4 ≥95%, CO_2 ≤2%, O_2 ≤0.3 % and negligible amounts of H_2S [3].

Conventional physical-chemical technologies such as water scrubbing, chemical scrubbing and membrane separation are commonly applied for CO_2 removal from biogas. However, these technologies often require a previous H_2S cleaning step such as activated carbon adsorption or chemical scrubbing [4]. On the contrary, biological H_2S removal technologies such as anoxic and aerobic biotrickling filters are not able to

remove CO_2 and present operational problems such as elemental sulfur accumulation (and subsequent clogging of the packed-bed) and biogas contamination with O_2 and N_2 [5, 6]. In addition, the physical-chemical technologies capable of simultaneously removing CO_2 and H_2S (for example chemical scrubbing with alkali aqueous solutions) exhibit high operating costs and a significant environmental impact [7].

In this regard, microalgae-based processes have emerged as a competitive and environmentally sustainable alternative for the simultaneous removal of CO_2 and H_2S from biogas [8]. These processes are based on the fixation of CO_2 via photosynthesis by microalgae and the oxidation of H_2S to sulfate by sulfur oxidizing bacteria using the oxygen photosynthetically produced. Moreover, the anaerobic effluents produced on-site can eventually support microalgae growth, thus reducing their associated treatment costs and eutrophication potential [9]. In addition, the algal biomass generated during the photosynthetic biogas upgrading process can be used as a feedstock for bio-fuel or bio-fertilizer production [10, 11], provided that biomass production has been properly maximized. However, the increase in pH and modification of metal ion speciation (e.g. Ca^{2+}, Mg^{2+} and Fe^{2+}) in the cultivation broth induced by microalgae growth can promote the abiotic removal of N and P by volatilization and precipitation, respectively [12]. This abiotic nutrient removal mechanism contributes to a detrimental loss of nutrients and causes a severe environmental impact derived from the indirect N_2O emissions associated to NH_4^+ stripping [13].

Several proof of concept studies of this innovative photosynthetic biogas upgrading process coupled with nutrient removal from digestate have been recently conducted by Bahr *et al*. [8], Serejo *et al*. [14] and Posadas *et al*., [15] in a HRAP interconnected to an

external CO_2−H_2S absorption column (AC). However, while a complete H_2S removal was always observed, CO_2 removal was low (<80%) and the upgraded biogas was contaminated with N_2 and O_2 (stripped out from the cultivation broth), the latter decreasing the CH_4 content in the upgraded biogas to ~80%. Therefore, the O_2 and N_2 content in the upgraded biogas represents nowadays the main limitation of this technology to achieve a high quality bio-methane, which entails the need to explore new operational strategies to minimize the desorption of these bio-methane pollutants from the algal-bacterial broth. In addition, little attention has been also paid to the optimization of nutrient recovery from digestates, which would enhance the environmental sustainability of the photosynthetic biogas upgrading process.

This research aimed at optimizing both the photosynthetic biogas upgrading process and nutrient recovery from digestate in an algal-bacterial HRAP interconnected to a biogas absorption column via recirculation of the settled broth. A preliminary optimization of the recycling liquid to biogas ratio was conducted in order to obtain a bio-methane with sufficient quality to be injected into natural grids. Then, an innovative HRAP operational strategy based on the control of algal-bacterial biomass productivity via settled biomass wastage was evaluated in order to enhance nutrient recovery from a synthetic digestate while producing a high quality bio-methane.

2. Materials and methods.

2.1 Experimental setup and operational conditions.

The experimental setup, located at the Dept. of Chemical Engineering and Environmental Technology at Valladolid University (Spain), consisted of a 180 L high rate algal pond (170 cm length × 82 cm width × 15 cm depth) with an illuminated area

of 1.21 m^2, interconnected to a 8 L conical settler and to a 2.2 L absorption column (4.4 cm diameter, 165 cm height) via recirculation of the settled algal cultivation broth (Figure 1). The HRAP was fed with a synthetic digestate at an influent flow rate of 1.3 ± 0.2 L m^{-2} d^{-1}, continuously agitated at an internal liquid recirculation velocity of ≈20 cm s^{-1}, and illuminated with fluorescent lamps at 420 ± 105 μmol m^{-2} s^{-1} using 16:8 h light:dark cycles. Tap water was supplied to compensate evaporation losses. The composition of the synthetic digestate was (mg L^{-1}): ammonium (NH_4^+) = 526 ± 132, total nitrogen (TN) = 646 ± 61, total phosphorous (TP) as P-PO_4^{3-} = 53 ± 11, inorganic carbon (IC) = 4458 ± 106 and sulfate (SO_4^{2-}) = 317 ± 83. Digestates are characterized by a high alkalinity and nutrient concentrations [16]. The effluent from the HRAP was collected in the settler and the clarified effluent was then pumped to the bottom of the AC at 1.6 m^3 m^{-2} h^{-1} (flow rates referred to the AC cross sectional area) co-currently with the biogas sparged (70% CH_4, 29.5% CO_2, 0.5% H_2S, Abello Linde (Barcelona, Spain)) through a metallic diffuser at 1.6 m^3 m^{-2} h^{-1}. The liquid phase exiting the AC was returned to the HRAP, while the excess of effluent from the system was removed by overflow from the settling tank. This innovative photobioreactor configuration allowed decoupling the hydraulic retention time from the algal bacterial biomass productivity by controlling the rate of settled biomass wasted and returned to the HRAP.

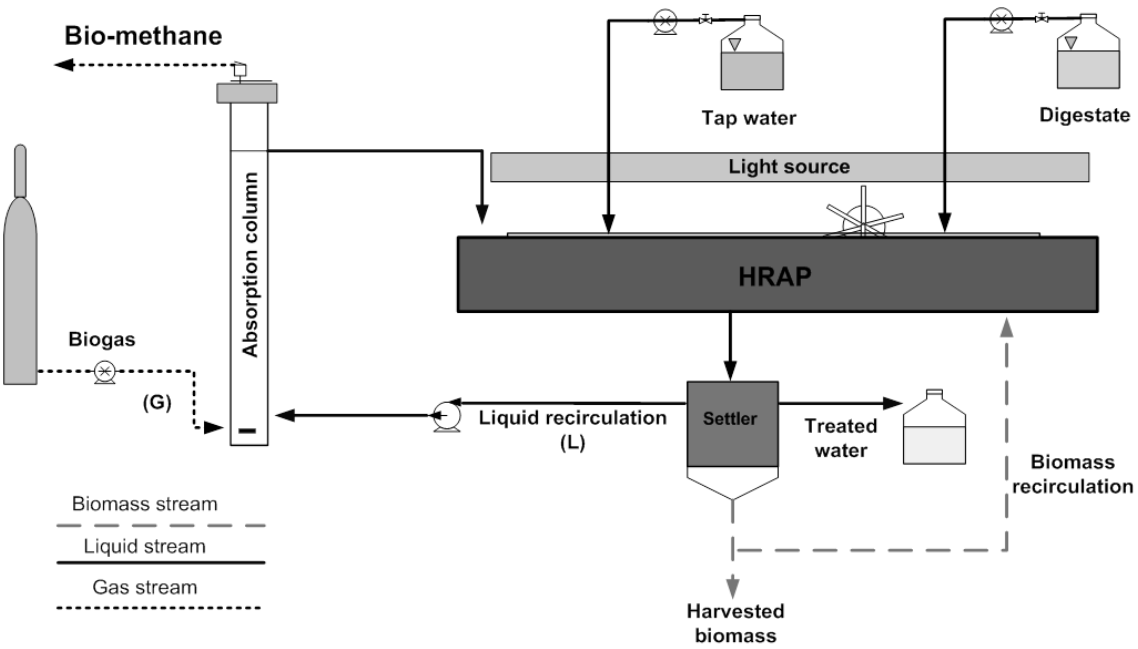


Figure 1. Schematic diagram of the experimental setup used for the continuous upgrading of biogas coupled to digestate treatment.

2.2 Influence of the recycling liquid to biogas ratio on the quality of the upgraded bio-methane.

L/G ratios ranging from 0.5 to 60 were tested in order to maximize CO_2 and H_2S removal while minimizing O_2 and N_2 desorption from the recycling liquid to the upgraded biogas. The synthetic biogas was sparged into the AC at 5.3, 16.0, and 31.5 mL min^{-1}, while the external liquid recirculation rate was set at 15, 60, 120, 203, and 315 mL min^{-1} for each biogas flow rate tested. The AC was constantly fed with the algal-bacterial broth at a pH of 10 ± 0.3. The absorption system was allowed to stabilize for two times the AC hydraulic retention time (HRT) prior to the monitoring of the upgraded biogas composition by GC-TCD.

2.3 Influence of biomass productivity on biogas upgrading and nutrient recovery.

155 The HRAP was inoculated with a consortium of cyanobacteria/microalgae composed of
156 *Geitlerinema* sp. (61.5%), *Staurosira* sp. (1.5%) and *Stigeoclonium tenue* (37%) from a
157 previous culture grown in diluted centrate wastewater. The consortium was then
158 acclimated to the digestate for 40 days prior to the experiment start-up. A biomass
159 productivity of 2.2 g m^{-2} d^{-1} was set during stage I (days 0-77) by controlling the rate of
160 withdrawal of settled biomass based on the total suspended solids (TSS) concentration
161 in the settler. The biomass productivity was increased to 4.4 g m^{-2} d^{-1} during stage II
162 (days 78-159) and to 7.5 g m^{-2} d^{-1} during stage III (days 160-202). The latter
163 productivity was selected based on the maximum biomass productivity expected from
164 the TP daily fed into the HRAP (assuming a P biomass content of 1 % according to
165 Alcántara *et al*., [17]). The experimental system was operated indoors for 202 days.
166 Liquid samples (100 mL) were collected twice a week from the digestate influent, the
167 treated digestate and the cultivation broth of the HRAP to monitor the pH and
168 concentration of IC, TN, NH_4^+, nitrite (NO_2^-), nitrate (NO_3^-), phosphate (PO_4^{3-}), SO_4^{2-}
169 and TSS. The TSS concentration of the settled biomass was also determined twice a
170 week to control biomass productivity. The temperature and dissolved O_2 concentration
171 (DO) were monitored in-situ. Gas samples from the inlet and outlet of the biogas
172 absorption column were periodically drawn to monitor the concentrations of CO_2, H_2S,
173 O_2, N_2, and CH_4. The inlet and outlet gas flow rates in the AC were also measured. An
174 aliquot of 50 ml of algal-bacterial biomass was taken in each steady state to characterize
175 the populations of microalgae and bacteria.
176
177 **2.4 Analytical procedures.**
178 Dissolved IC and TN concentrations were determined using a Shimadzu TOC-VCSH
179 analyzer (Japan) equipped with a TNM-1 chemiluminescence module. NH_4^+ was

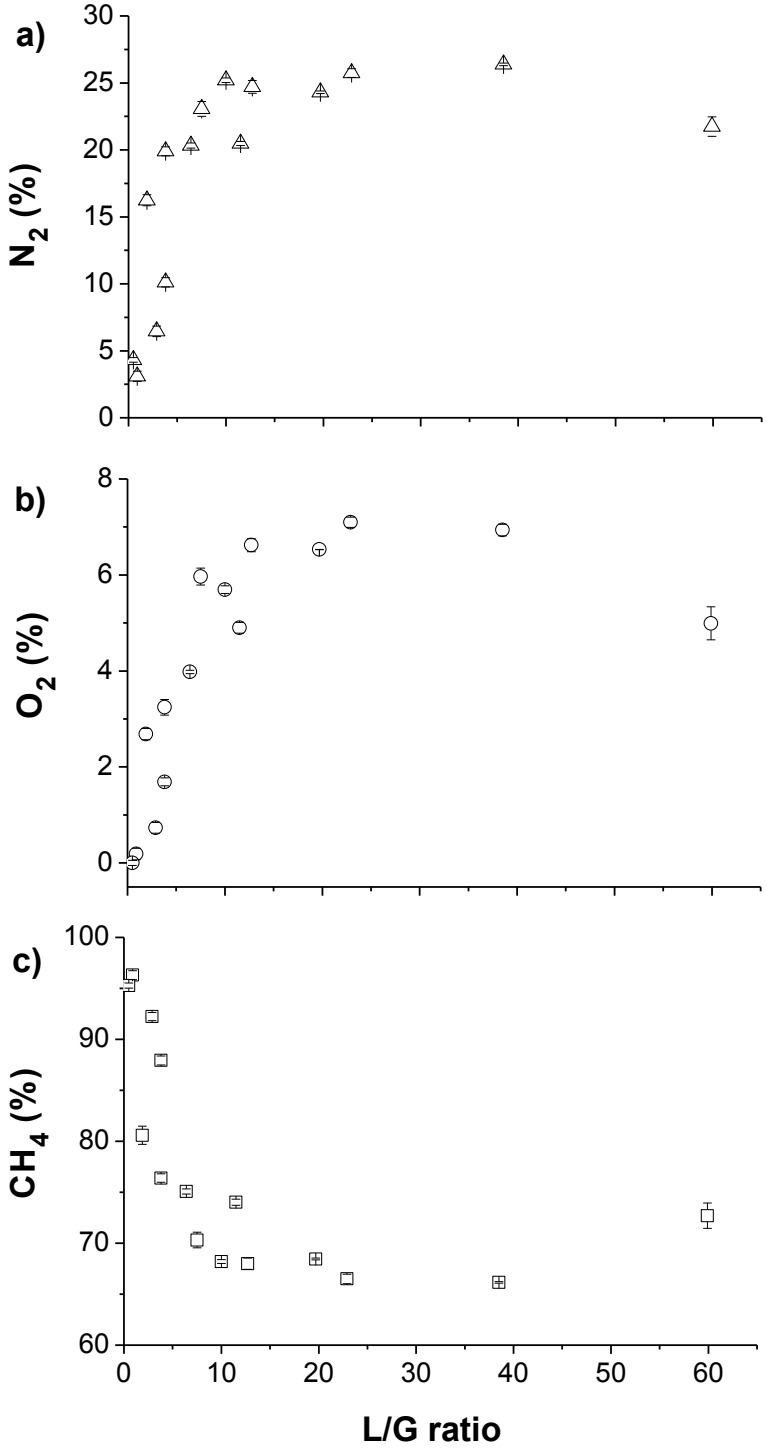
measured using an ammonia electrode Orion Dual Star (Thermo Scientific, The Netherlands). NO_3^-, NO_2^-, PO_4^{3-} and SO_4^{2-} concentrations were analyzed by HPLC-IC according to Serejo *et al.*, [14]. TSS analyses were carried out according to Standard Methods [18]. The pH in the cultivation broth was monitored with a pH meter Eutech Cyberscan pH 510 (Eutech instruments, The Netherlands). The light intensity at the HRAP surface was measured with a LI-250A light meter (LI-COR Biosciences, Germany). The C and N contents of the algal-bacterial biomass were determined using a CHNS analyser (LECO CHNS-932), while P and S contents were determined using an Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES, Varian 725-ES) after microwave-acid digestion [19]. The biogas CO_2, H_2S, O_2, N_2, and CH_4 concentrations were analyzed by GC-TCD according to Posadas *et al.*, [15]. The morphological identification of microalgae was carried out by microscopic observations (OLYMPUS IX70, USA) after sample fixation with 5% of lugol acid. The bacterial community determination was conducted by DGGE-sequencing according to Posadas *et al.*, [15] and the sequences were deposited in GenBank Data Library under accession numbers KU605583-KU605606.

3. Results and discussion.

3.1 Influence of the recycling liquid to biogas ratio on the quality of the upgraded biogas.

The performance of the photosynthetic biogas upgrading process can be optimized by determining the optimum L/G ratio in order to prevent O_2 and N_2 desorption while boosting the absorption of CO_2 and H_2S. CO_2 mass transfer from the biogas is a function of pH, CO_2 concentration, temperature, pressure and ionic strength of the recycling algal-bacterial broth. Since H_2S and CO_2 are acidic gases, a more efficient

absorption of these biogas pollutants would be expected at a high pH. In our particular study, the pH of the algal-bacterial broth was 10, which supported CO_2 and H_2S removal efficiencies (REs) of 98.8 ± 0.19% and 97.1 ± 1.4%, respectively, regardless of the L/G ratio tested. However, the N_2 and O_2 stripped from the cultivation broth increased linearly at increasing the L/G ratio, to finally stabilize at 25% and 7%, respectively (Figures 2a and 2b). In contrast to the results here obtained, Serejo *et al*., [14] reported a stabilization in the CO_2-REs at 95 ± 2% at L/G ratios above 15 (likely due to the relatively low pH of the cultivation broth ≈7.9), with a maximum O_2 concentration in the upgraded biogas of 3 ± 1%. The higher N_2 and O_2 concentrations here observed were likely due to the increase in the overall mass transfer coefficients in the AC as a result of the higher ionic strength of the cultivation broth (IC =2300 mg L^{-1}), which prevented the coalescence of the fine bubbles produced by the diffuser. This increased contamination of the upgraded biogas at increasing L/G ratios resulted in a concomitant decrease in CH_4 concentration from 95% at a L/G of 1 down to 68% at L/G >15. Similar results were reported by Posadas *et al*. [15], who despite the high CO_2 and H_2S REs obtained, observed a decrease in the final CH_4 concentration down to 81 ± 2% as a result of a high N_2 content in the upgraded biogas. Hence, a L/G ≤1 resulted in CH_4 concentrations over 95% (Figure 2c), and in H_2S, CO_2, O_2 and N_2 concentrations lower than 0.007%, 0.4%, 0.2% and 3%, respectively, which complied with most European bio-methane legislations. Therefore, the recycling liquid to biogas ratio was identified as a key operating factor determining the final quality of the upgraded biogas.



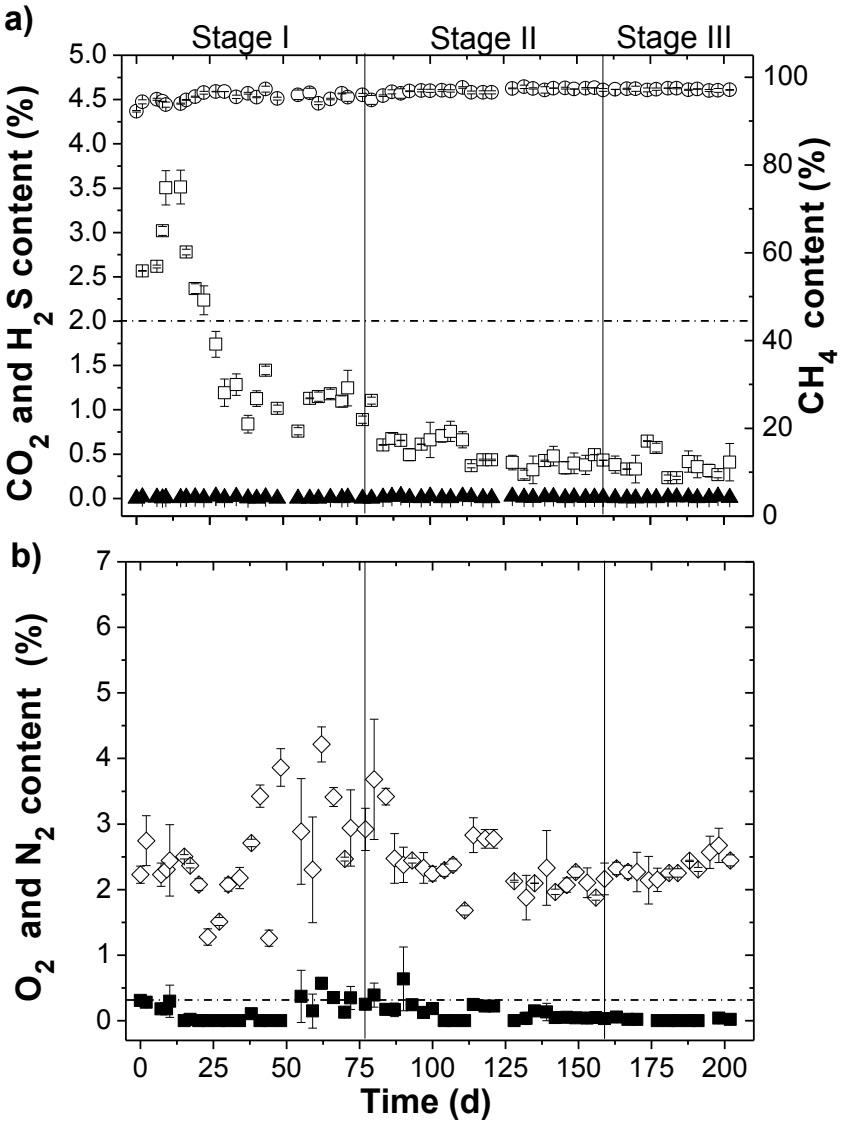
226

227 **Figure 2.** Influence of the recycling liquid to biogas ratio on the concentrations of (a)
228 N_2, (b) O_2 and (c) CH_4 in the upgraded biogas. Vertical bars represent the standard
229 deviation from replicate measurements.

230

231 **3.2 Influence of biomass productivity on biogas upgrading to bio-methane.**

The CO_2 content in the bio-methane gradually decreased during stage I from 3.5% to 1.2% (Figure 3a), concomitantly with the increase in the pH of the cultivation broth in the HRAP up to 9.1 ± 0.1 due to microalgal photosynthetic activity (Table 1). These results confirmed that the high CO_2-REs here recorded significantly depended on the pH of the cultivation broth. In spite of the high CO_2 absorption recorded in the AC, only a slight decrease in the pH (0.1-0.3 gradient) from the bottom to the top of the AC was observed. These results were not in agreement with those reported by Meier *et* al., [20] using a similar two-stage system, who observed a pH gradient of ~1-2 along the column depending on the cultivation broth recycling rate. This difference was attributed to the high buffer capacity of the digestate used in this study for microalgae growth. The average steady CO_2-REs obtained in stages I, II and III were 96.6 ± 1.2%, 98.4 ± 0.8% and 99 ± 0.3%, respectively (Figure 4). The increase in the pH of the cultivation broth from 9.1 to 10.6, likely mediated by the increase in the overall photosynthetic activity (which itself was induced by the increase in biomass productivity), supported the higher CO_2-REs recorded. These values were higher than those recorded by Bahr *et al*. [8] (RE = 86 ± 5%) using a similar experimental setup operated with a highly carbonated mineral salt medium at a pH of 9.4 and at a L/G ratio of 1. On the other hand, despite similar CO_2-REs (97%) from a synthetic biogas containing 41% of CO_2 were reported by Mann *et al.* [21], contamination of the upgraded biogas with up to 23.4% of O_2 was also observed in their study.



253 **Figure 3.** Time course of the concentration of (a) CO_2, (□) H_2S (▲) and CH_4 (○), and
254 b) oxygen (■) and nitrogen (◊) in the upgraded biogas. The horizontal dashed lines
255 indicate the maximum CO_2 and O_2 concentrations required for bio-methane injection
256 into natural gas grids. Vertical bars represent standard deviation from replicate
257 measurements.

258

259 An almost complete H_2S removal was recorded regardless of the biomass productivity
260 set: 99.0 ± 1.0%, 98.0 ± 1.2% and 98.5 ± 1.0% in stages I, II and III, respectively
261 (Figure 4), which was in accordance with those reported by Serejo *et al*. [14] and
262 Posadas *et al*. [15]. Sulfate formation was observed as a result of the biological H_2S

oxidation. At this point it must be highlighted that no oxygen limitation occurred throughout the entire experimentation. Dissolved oxygen concentration increased from 5.4 ± 0.8 mg O_2 L^{-1} in stage I to 9.6 ± 0.4 mg O_2 L^{-1} in stage III as a result of the increase in microalgae productivity. The sulfate concentrations during stages I, II and III were 388 ± 43 mg-SO_4^{2-} L^{-1} , 483 ± 24 mg-SO_4^{2-} L^{-1} and 386 ± 52 mg-SO_4^{2-} L^{-1}, respectively. The decrease observed during stage III was attributed to the increase in biomass productivity (sulfate assimilation into biomass). The sulfur mass balance revealed that only 40% of the sulfur removed was oxidized to sulfate, the remaining 60% being likely present (dissolved or in suspension) as S-intermediates such as S^o, thiosulfate or sulfite. Partial oxidation of the H_2S transferred from biogas has been previously reported [22], however a further analysis of the sulfur compounds present in the cultivation broth is necessary in order to elucidate the fate of the H_2S removed.

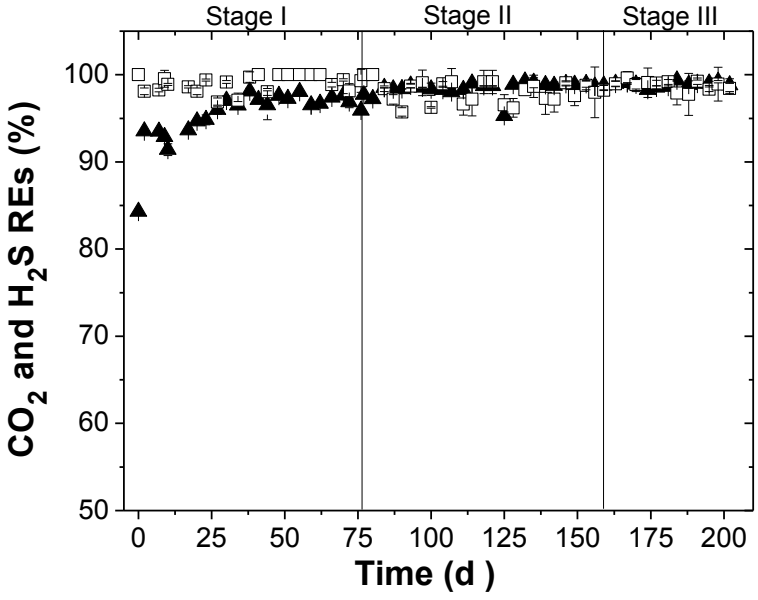


Figure 4. Time course of the removal efficiencies of CO_2 (▲) and H_2S (□). Vertical bars represent standard deviation from replicate measurements.

The biological oxidation of CH_4 resulted in average CH_4 losses of 4.9 ± 2.4% (on a mass basis) during stage I, no methane losses being recorded afterwards. The CH_4 content in the upgraded biogas was 95.8 ± 0.8%, 96.9 ± 0.7% and 97.2 ±0.2% in stages I, II and III, respectively (Figure 3a). These values are comparable to those achieved by water scrubbing technologies, where CH_4 losses by dissolution in the pressurized water of 3-5% result in CH_4 purities of 80-99%, depending on the N_2 and O_2 content of the upgraded biogas [23].

The O_2 demand in the absorption column resulting from the biological oxidation of H_2S caused an oxygen content in the upgraded biogas of 0.1 ± 0.2% in stages I and II and 0.03 ± 0.04% in stage III (Figure 3b). These O_2 concentrations recorded in the bio-methane were lower than the values obtained by Meier *et al*. [20] (1.2%) and Posadas *et al*., [15] (0.7-1.2%), and remained significantly below those reported in literature during biogas upgrading in algal photobioreactors (10-24%) [21, 24]. Finally, the N_2 stripped out from the recycling cultivation broth resulted in average concentrations of 2.6 ± 0.9%, 2.4 ± 0.5% and 2.4 ± 0.2% during stages I, II and III, respectively (Figure 3b), due to the low L/G ratio applied in this study (which limited the amount of N_2 potentially desorbed). Higher N_2 concentrations in the upgraded biogas of 6-8% were recorded by Posadas *et al*., [15] and Serejo *et al*., [14] during photosynthetic biogas upgrading at a L/G of 10. In this context, the optimum bio-methane composition was obtained at the highest microalgae productivity evaluated (0.4 ± 0.1% CO_2, 0.03 ± 0.04% O_2, 2.4 ± 0.2% N_2 and 97.2 ± 0.2% CH_4), which complied with the regulatory limits of most European legislations for bio-methane injection in natural gas grids (Figures 3a and 3b). For instance, the injection of bio-methane into the Spanish network

allows up to 0.3% of O_2 provided that CO_2 concentration does not exceed 2% and CH_4 concentration remains over 95% [25].

3.3 Influence of biomass productivity on nutrient removal and nutrient recovery.

Most recent life cycle analyses have shown that the use of wastewater as a low-cost nutrients and water source can reduce the overall energy requirements and improve the environmental sustainability of microalgae mass production [26, 27]. In our particular case, microalgae production using the N and P present in anaerobically digested wastewaters can significantly decrease the operating costs of the biogas upgrading process, while preserving fresh water resources and recovering these nutrients in the form of a microalgae biomass that can be further valorized as a bio-fertilizer. Despite the potential of microalgal biotechnology to fix nutrients from digestates, abiotic removal still represents an important mechanism for nutrient removal from wastewater in algal-bacterial processes. Thus, N removal by stripping can account for up to 82% [28] and P removal by precipitation for up to 63% [29] of the total nutrients supplied. Nonetheless, the monitoring of this abiotic nutrient removal in HRAPs is often disregarded [12].

Table 1. Average dissolved oxygen concentration, pH, temperature, total suspended solid concentration and biomass productivity recorded during the three operational stages.

Stage	T_{HRAP} (ºC)	PH_{HRAP}	DO (mg O_2 L^{-1})	TSS HRAP (g L^{-1})	Productivity (g m^{-2} d^{-1})
I	22 ± 3	9.1 ± 0.1	5.4 ± 0.8	1.6 ± 0.1	2.2 ± 1.4
II	25 ± 2	9.6 ± 0.3	7.5 ± 1.4	1.2 ± 0.4	4.4 ± 1.5
III	28 ± 1	10.6 ± 0.1	9.6 ± 0.4	0.9 ± 0.1	7.5 ± 0.1

323 The high buffer capacity of the cultivation broth as a result of the high IC
324 concentrations present in the digestate and the high water evaporation losses, together
325 with the high photosynthetic activity in the system, maintained high pH values during
326 the three operational stages without an automatic pH control (Table 1). The temperature
327 of the algal–bacterial broth slightly increased concomitantly with the seasonal variation
328 of the ambient temperature, but remained close to optimum values for microalgae and
329 bacteria cultivation. Apart from the impinging radiation, other variables such as the
330 nutrients load (determined by the flow rate and nutrients concentration of the target
331 wastewater) and biomass concentration in the cultivation broth (determining light
332 penetration) influence microalgae productivity in HRAPs devoted to wastewater
333 treatment. For instance, low biomass concentrations (~0.5 g L^{-1}) are typically
334 encountered in open ponds treating domestic wastewaters at HRTs of 5-10 days.
335 Biomass productivity can be thus boosted by increasing the nutrients load into the
336 HRAPs, provided that light supply does not limit the process. However, while an
337 increase in wastewater flow rate might induce microalgae washout, the use of
338 wastewaters with high nutrient concentrations (such as digestates) would entail very
339 dense microalgae cultures, which would ultimately limit microalgae productivity as a
340 result of an excessive mutual shading. In this context, the decoupling between the
341 hydraulic retention and biomass retention time (inversely related to microalgae
342 productivity) represents an innovative strategy for maximizing biomass productivity
343 during microalgae cultivation in high-strength wastewaters. The control of biomass
344 productivity via regulation of the settled biomass wastage rate would allow maximizing
345 nutrient recovery from wastewaters. A TSS concentration of 1.6 ± 0.1 g L^{-1} was
346 recorded in the HRAP when operating at an average productivity of 2.2 g m^{-2} d^{-1} in
347 stage I. This TSS concentration decreased to 1.2 ± 0.4 g L^{-1} and 0.9 ± 0.1 g L^{-1} under

348 operation at 4.4 g m^{-2} d^{-1} and 7.5 g m^{-2} d^{-1}, respectively. The results clearly showed that
349 an increase in the rate of biomass wastage from the settler resulted in lower TSS
350 concentrations, which likely improved the overall photosynthetic efficiency as a result
351 of an enhanced light penetration. In addition, the control of biomass productivity was
352 supported by the good settling properties of the algal-bacterial biomass present in the
353 HRAP. However, a decrease in the TSS removal efficiency of the settler from 95 ± 3%
354 in stage I to 84 ± 4% in stage III was recorded, which was attributed to the shift in
355 microalgae population observed in stage II (see section 3.4). Unfortunately, the effluent
356 TSS concentrations (70 ± 50 mg L^{-1}) remained always over the maximum discharge
357 limit in European Union legislation (35 mg L^{-1}) [30].
358

Table 2. Average removal efficiencies of total nitrogen, ammonium, phosphorus, inorganic carbon and total suspended solids recorded during the three operational stages.

Stage	Removal efficiencies (%)				
	TN	$N\text{-}NH_4^+$	$P\text{-}PO_4^{3-}$	IC	TSS
I	91 ± 4	100	77 ± 16	86 ± 6	95 ± 3
II	92 ± 4	100	63 ± 18	78 ± 10	91 ± 10
III	98 ± 2	100	73 ± 19	70 ± 9	84 ± 4

359

360 A complete removal of ammonium was observed during all stages, while TN-REs
361 increased from 91 ± 4% up to 98 ± 2% when biomass productivity increased from 2.2 to
362 7.5 g m^{-2} d^{-1} (Table 2). Despite the slight influence of biomass productivity on TN-REs,
363 the share of the inlet TN assimilated into biomass varied from 19 ± 13 % at the lowest
364 microalgae productivity to 83 ± 9% at the highest productivity (Table 3). In this context,
365 the low nitrification activity recorded along with the high pH value supported a
366 significant $N\text{-}NH_4^+$ removal by stripping, which decreased from 75 ± 12% in stage I to
367 13 ± 9% in stage III. On the other hand, phosphorus removal remained stable regardless
368 of the biomass productivity set, with REs of 77 ± 16%, 63 ± 18% and 73 ± 19% in

369 stages I, II and III, respectively. Serejo *et al*. [14] recorded similar phosphorous REs (71
370 ± 3%) at a comparable biomass productivity (7.1 ± 0.8 g m^{-2} d^{-1}) during the treatment of
371 anaerobically digested vinasse coupled to biogas upgrading. Similar to the share of TN
372 assimilated, the increase in biomass productivity resulted in an increase in the
373 contribution of P assimilation to the TP removal from 22 ± 12% to 100%. The absence
374 of PO_4^{3-} volatilization, together with the high pH prevailing in the cultivation broth
375 throughout the entire experimental period, suggested that precipitation was the main
376 phosphorous removal mechanism under low biomass productivities. Therefore, the
377 control of biomass productivity via regulation of the biomass wastage rates allowed
378 maximizing nutrient recovery in the form of algal biomass in detriment of the abiotic
379 nutrients removal mechanisms.
380

Table 3. Nutrient recovery via biomass assimilation estimated from the nutrient removed and the elemental composition and mass flow rate of the biomass harvested during the three operational stages.

Stage	Nutrient recovery as biomass (%)			Biomass elemental composition (%)		
	C	P	N	C	P	N
I	6 ± 3	22 ± 12	19 ± 13	43.6	0.7	6.5
II	16 ± 5	50 ± 19	36 ± 18	46.5	0.8	7.2
III	30 ± 1	100	83 ± 9	48.0	0.9	6.7

381

382 **3.4 Consortia of cyanobacteria/microalgae and bacteria.**

383 The microalgae and cyanobacteria species initially present in the inoculum were
384 gradually replaced along the three operational stages. The cyanobacterium prevailing in
385 the inoculum (*Geitlerinema* sp.) was not observed under steady state conditions in
386 stages I, II and III. Thus, the cyanobacteria/microalgae consortium was mainly
387 composed of *Limnothrix planktonica* (32.9%), *Acutodesmus obliquus* (2.6%), *Chlorella*
388 *vulgaris* (2.6%), *Mychonastes homosphaera* (5.9%), *Navicula* sp. (0.7%), *Phormidium*
389 sp. (19.7%) and *Stigeoclonium tenue* (35.5%) during stage I. This high diversity was

similar to that reported in indoor HRAP treating digestates [14, 15]. Surprisingly, this high microalgae diversity disappeared in stage II with the establishment of an unialgal culture of the Chlorophyta *Mychonastes homosphaera*. This unialgal culture remained dominant throughout stage III likely due to the extreme environmental conditions prevailing in this study (high pH and salinity as a result of the high water evaporation losses). In addition, the control of biomass productivity via regulation of the settled biomass wastage rate applied might have also influenced the dominant species and algal/bacterial ratio since the increase in biomass productivity likely induced the development of fast growing microorganisms. *Mychonastes homosphaera* (Skuja) Kalina & Puncochárová is currently regarded as a taxonomic synonym of *Chlorella minutissima* Fott & Nováková. The potential of this microalga for wastewater treatment [31], heavy metal removal [32] and biodiesel production has been consistently demonstrated, *Mychonastes homosphaera* being capable of storing a desirable fatty acid profile under nitrogen starvation [33]. The valorization of this microalga into high-added value chemicals or biofuels such as syngas, bioethanol or bio-oil using a biorefinery approach will certainly enhance the sustainability and economic viability of microalgae-based biogas upgrading [34].

The high diversity revealed by microscopic observation was confirmed by the Shannon-Wiener diversity indexes obtained, which ranged from 1.5 to 3.5 (Figure 1, supplementary material). The slight decrease of this index from 3.2 in stage I to 2.9 in stage II also confirmed the shift in algae diversity microscopically observed. Likewise, the analysis of the Pearson similarity coefficients showed a high similarity between the microbial communities present in stages II and III (99%), which was in agreement with the above mentioned establishment of a dominant microalga specie. The DGGE analysis (Figure 1, supplementary material) showed 24 bands, which were sequenced. Six

415 different phyla were retrieved from the RDP database: Cyanobacteria/Chloroplast (10
416 bands), *Acidobacteria* (4 bands), *Proteobacteria* (4 bands), *Deinococcus-thermus* (1
417 band), *Chloroflexi* (1 band), *Actinobacteria* (1 band) (Table 1, supplementary material).
418 The morphological identification of *Mychonastes homosphaera* was confirmed by
419 bands 8, 9 and 10, which belonged to the genus Chlorophyta and were related to
420 *Chlorella* species. The phyla *Acidobacteria* (bands 12 and 13), *Proteobacteria* (bands
421 15 and 16) and *Actinobacteria* (band 21) were found in the three operational stages,
422 while the phylum *Chloroflexi* was detected in the inoculum and stages II and III.
423 Bacteria from the genus *Blastocatella* (band 11) and the *Gammaproteobacteria* class
424 (band 15), which have been identified in activated sludge [35] and HRAPs treating
425 piggery wastewater [36], respectively, likely supported the aerobic biodegradation of
426 the organic matter and ammonia contained in the digestate. Finally, the identification of
427 the genus *Thioalbus* (band 16) confirmed the biological nature of H_2S oxidation [37].
428 To the best of our knowledge, this is the first time that sulfur-oxidizing bacteria
429 (facultative microorganisms that can use O_2 or NO_3^- as electron acceptors) have been
430 found in these photosynthetic biogas upgrading processes.
431
432 **3.5 Conclusions.**
433 This study confirmed the potential of photosynthetic biogas upgrading to support a cost-
434 efficient bio-methane production coupled to nutrient recovery from digestate. To the
435 best of our knowledge, this is the first experimental study reporting biological biogas
436 upgrading to a bio-methane complying with most European legislations for biogas
437 injection into natural gas grids. An almost complete removal of H_2S and CO_2, and
438 concentrations of O_2 and CH_4 in the upgraded biogas <0.1% and >95%, respectively,
439 were achieved regardless of the biomass productivity set. The innovative HRAP

operational strategy here developed allowed enhancing nutrient recovery by shifting from an abiotic-based nutrients removal to an assimilatory-based removal. Furthermore, the extreme cultivation conditions established in the HRAP expedited the dominance of the microalga *Mychonastes homosphaera*, and supported the growth of sulfur-oxidizing bacteria. The presence of sulfur-oxidizing bacteria from the genus *Thioalbus* confirmed, for the first time, the biological nature of H_2S oxidation during biogas upgrading in algal-bacterial photobioreactors.

References.

[1] EurObserv'ER; EurObserv'ER Website. Biogas Barometer; 2014; http://www.energies-renouvelables.org/observ-er/stat_baro/observ/baro224_Biogas_en.pdf. (Last accessed, 02/02/2016)

[2]. Ryckebosch, E.; Drouillon, M.; Vervaeren, H. Techniques for transformation of biogas to bio-methane. Biomass Bioenerg. **2011**, 35, 1633-1645

[3] Muñoz, R.; Meier, L.; Díaz, I.; Jeison, D. A review on the state-of-the-art of physical/chemical and biological technologies for biogas upgrading. Rev Environ Sci Biotechnol**. 2015**, 14 (4), 727-759

[4] International Energy Agency. 2013. https://www.iea.org/topics/bioenergy/

[5] Fortuny, M.; Baeza, J.A.; Gamisans, X.; Casas, C.; Lafuente, J.; Deshusses, M.A.; Gabriel, D. Biological sweetening of energy gases mimics in biotrickling filters. Chemosphere. **2008**, 71(1), 10-17

[6] Montebello, A.M.; Fernández, M.; Almenglo, F.; Ramírez, M.; Cantero, D.; Baeza, M.; Gabriel, D. Simultaneous methylmercaptan and hydrogen sulfide removal in the desulfurization of biogas in aerobic and anoxic biotrickling filters. Chem. Eng. J. **2012**, 200, 237-246

[7] Tippayawong, N.; Thanompongchart, P. Biogas quality upgrade by simultaneous removal of CO_2 and H_2S in a packed column reactor. Energy. **2010**, **35** (12), 4531-4535

[8] Bahr, M.; Díaz, I.; Domínguez, A.; González Sánchez, A.; Muñoz, R. Microalgal-biotechnology as a platform for an integral biogas upgrading and nutrient removal from anaerobic effluents. Environ. Sci. Technol. **2014**, 48, 573-581

[9] Uggetti, E.; Sialve, B.; Latrille, E.; Steyer, J-P. Anaerobic digestate as substrate for microalgae culture: The role of ammonium concentration on the microalgae productivity. Bioresour. Technol. **2014**, 152, 437-443

[10] Mulbry, W.; Westhead, E.K.; Pizarro, C.; Sikora, L. Recycling of manure nutrients: use of algal biomass from dairy manure treatment as a slow release fertilizer. Bioresour. Technol. **2006**, 96(4), 451-458

[11] Razzak, S.A.; Hossain, M.M.; Lucky, R.A.; Bassi, A.S.; de Lasa, H. Integrated CO_2 capture, wastewater treatment and biofuel production by microalgal culturing-A review. Renew. Sust. Energ. Rev. **2013**, 27, 622-653

[12] Whitton, R.; Ometto, F.; Pidou, M.; Jarvis, P.; Villa, R.;Jefferson. B. Microalgae for municipal wastewater nutrient remediation: mechanisms, reactors and outlook for tertiary treatment. Environ. Technol. Rev. **2015**, DOI: 10.1080/21622515.2015.1105308

[13] Pratt, C.; Redding, M.; Hill, J.; Shilton, A.; Chung, M.; Guieysse, B. Good science for improving policy: greenhouse gas emissions from agricultural manures. Anim. Prod. Sci. **2014**, 55 (6) Pages 691-701

[14] Serejo, M.; Posadas, E.; Boncz, M.; Blanco, S.; García-Encina, PA.; Muñoz, R. Influence of biogas flow rate on biomass composition during the optimization of biogas upgrading in microalgal-bacterial processes. Environ. Sci. Technol. **2015**, 49, 3228-3236

489 [15] Posadas, E.; Serejo, M.; Blanco, S.; Pérez, R.; García-Encina, P.A.; Muñoz, R.
490 Minimization of bio-methane oxygen concentration during biogas upgrading in algal-
491 bacterial photobioreactors. Algal Research. **2015**, 12, 221-229
492 [16] Wilkie, A.C.; Riedesel, K.J.; Owens, J.M. Stillage characterization and anaerobic
493 treatment of ethanol stillage from conventional and cellulosic feedstocks. Biomass
494 Bioenerg. **2000**, 19, 63-102
495 [17] Alcántara, C.; García-Encina, P.A.; Muñoz R. Evaluation of mass and energy
496 balances in the integrated microalgae growth-anaerobic digestion process. Chem. Eng.
497 J. **2013**, 221 (1), 238-246
498 [18] Eaton AD, Clesceri LS, Greenberg AE. 2005. Standard methods for the
499 examination of water and wastewater. 21st ed.; Washington, DC, 2005
500 [19] Alcántara, C.; García-Encina, P.A.; Muñoz, R. Evaluation of the simultaneous
501 biogas upgrading and treatment of centrates in a high-rate algal pond through C, N and
502 P mass balances. Water Sci Technol. **2015**, 72(1), 150-157
503 [20] Meier, L.; Pérez, R.; Azócar, L.; Rivas, M.; Jeison, D. Photosynthetic CO_2 uptake
504 by microalgae: An attractive tool for biogas upgrading. Biomass Bioenerg. **2015**, 73,
505 102-109
506 [21] Mann, G.; Schlegel, M.; Sakalauskas, R.S.A. Biogas-conditioning with microalgae.
507 Agron. Res. **2009**, 7, 33-38
508 [22] Lebrero, R.; Toledo-Cervantes, A.; Muñoz, R.; Nery, V.; Foresti, E. Biogas
509 upgrading from vinasse digesters: a comparison between an anoxic biotrickling filter
510 and an algal-bacterial photobioreactor. J. Chem. Technol. Biot. **2015**, DOI:
511 10.1002/jctb.4843

512 [23] Sun, Q.; Li, H.; Yan, J.; Liu, L.; Yu, Z.; Yu, X. Selection of appropriate biogas
513 upgrading technology-a review of biogas cleaning, upgrading and utilization. Renew.
514 Sust. Energ. Rev. **2015**, 51, 521-532
515 [24] Converti, A.; Oliveira, R.P.S.; Torres, B.R.; Lodi, A.; Zilli, M. Biogas production
516 and valorization by means of a two-step biological process. Bioresour. Technol. **2009**,
517 100, 5771-5776
518 [25] BOE. Boletin oficial del Estado. 2013; cve: BOE-A-2013-185
519 [26] Sturm, B.; Lamer, S.L. An energy evaluation of coupling nutrient removal from
520 wastewater with algal biomass production. Appl. Energ. **2011**, 88(10), 3499-3506
521 [27] Pittman, J.K.; Dean, A.P.; Osundeko, O. The potential of sustainable algal biofuel
522 production using wastewater resources. Bioresour. Technol. **2011**, 102(1), 17-25
523 [28] Martínez, M.E.; Sánchez, S.; Jimenez, J.M.; El Yousfi, F.; Muñoz, L. Nitrogen and
524 phosphorus removal from urban wastewater by the microalga Scenedesmus obliquus.
525 Bioresour. Technol. **2000**, 73(3), 263-272
526 [29] Larsdotter, K.; La Cour, J.J.; Dalhammar, G. Biologically mediated phosphorus
527 precipitation in wastewater treatment with microalgae. Environ. Technol. **2007**, 28(9),
528 953-960
529 [30] European Directive 91/271/CEE on discharge of domestic wastewaters.
530 [31] Bhatnagar, A.; Bhatnagar, M.; Chinnasamy, S.; Das, K.C. *Chlorella minutissima*.
531 A Promising Fuel Alga for Cultivation in Municipal Wastewaters. Appl. Biochem.
532 Biotech. **2010**, 161(1), 523-536
533 [32] Yang, J.; Cao, J.; Xing, G.; Yuan, H. Lipid production combined with biosorption
534 and bioaccumulation of cadmium, copper, manganese and zinc by oleaginous
535 microalgae *Chlorella minutissima* UTEX2341. Bioresour. Technol. **2015**, 175, 537-544

536 [33] Tang, H.; Chen, M.; Garcia, M.E.D.; Abunasser, N.; Ng K.Y.S.; Salley S.O.
537 Culture of microalgae *Chlorella minutissima* for biodiesel feedstock production.
538 Biotechnol. Bioeng. **2011**, 108(10), 2280-2287
539 [34] Toledo-Cervantes, A.; Morales, M. Biorefinery: Using microalgal biomass for
540 producing energy and chemicals. In Energy and Environment Nowadays. Torres, L.G.,
541 Bandala, E.R.; Ed. Nova publisher, Nova Science Publishers, Inc. NY, USA. 2014; pp
542 19-56
543 [35] Layton, A.C.; Karanth, P.N.; Lajoie, C.A.; Meyers, A.J.; Gregory, I.R.; Stapleton,
544 R.D.; Taylor, D.E.; Sayler, G. S. Quantification of *Hyphomicrobium* Populations in
545 Activated Sludge from an Industrial Wastewater Treatment System as Determined by
546 16S rRNA Analysis. Appl. Environ. Microb. **2000**, 66(3), 1167-1174
547 [36] Ferrero, E.M.; de Godos, I.; Rodríguez, E.M.; García-Encina, P.A.; Muñoz, R.;
548 Bécares, E. Molecular characterization of bacterial communities in algal–bacterial
549 photobioreactors treating piggery wastewaters. Ecol. Eng. **2012**, 40, 121-130
550 [37] Park, S.J.; Pham, V.H.; Jung, M.Y.; Kim, S.J.; Kim, J.G.; Roh, D.H.; Rhee, S.K.
551 *Thioalbus denitrificans* gen. nov., sp. nov., a chemolithoautotrophic sulfur-oxidizing
552 gammaproteobacterium, isolated from marine sediment. Int. J. Syst. Evol. Micr. **2011**,
553 61, 2045 -2051

Acknowledgments.

This research was supported by MINECO (CTM2015-70442-R and Red Novedar), the Regional Government of Castilla y León (Project VA024U14 and UIC 71) and INIA (RTA2013-00056-C03-02). CONACyT-México is also gratefully acknowledged for the Postdoctoral grant of Alma Toledo (No. Reg: 237873).

Supplementary Material

Click here to download Supplementary Material: supplementary material Manuscript Toledo-Cervantes_ 2016.doc

Graphical Abstract