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EVALUATION OF MASS AND ENERGY BALANCES IN THE COMBINED MICROALGAE GROWTH-DIGESTION PROCESS

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Que Dña. Cynthia Alcántara Pollo ha realizado bajo su dirección el Trabajo Fin de Master, del Master en Investigación en Ingeniería de Procesos y Sistemas, titulado EVALUATION OF MASS AND ENERGY BALANCES IN THE COMBINED MICROALGAE GROWTH-DIGESTION PROCESS.

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Valladolid, a 5 de julio de 2012

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EVALUATION OF MASS AND ENERGY BALANCES IN THE COMBINED MICROALGAE GROWTH-DIGESTION PROCESS

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ABSTRACT

The fundamental mass and energy balances of the combined growth-digestion process are necessary to quantify the potential for energy and nutrients recovery in the overall biofuel production process from microalgae. This study quantified the C, N, and P mass balances and the energy balance in the integrated process of microalgae photosynthetic cultivation (under autotrophic and mixotrophic conditions) followed by anaerobic digestion. Under fully photoautotrophic growth, the biomass stoichiometric formula was $CH_{1,63}N_{0,14}O_{0,43}P_{0,006}S_{0,005,}$ while the maximum photosynthetic efficiency (PE) and microalgae production rate accounted for 7,6% and 71,3 $g/m^3 \cdot d$, respectively. *C. sorokiniana* was capable of photoautotrophically assimilating 1,8 ± 0,1 g of CO_2 per g of TSS of microalgae and released 1,0 ± 0.0 g of O_2 per g of CO_2 consumed. The stoichiometric formula of microalgae mixotrophically grown was $CH_{1,68}N_{0,16}O_{0,48}P_{0,006}S_{0,008}$, with only 13,8 ± 1,2% of the initial C-glucose present in the MSM assimilated, which suggested a partial growth inhibition by glucose in the particular MSM here used. Mixotrophic conditions supported a specific growth rate at the exponential growth phase (μ) of 1,6 d^{-1}, similar to 1,5 d^{-1} under autotrophic conditions, and reached a maximum PE of 7,4% and a maximum production rate of 142,3 $g/m^3 \cdot d$. The anaerobic digestion of microalgae cultivated autotrophically, showed that 54,6% ± 0,6 of the initial C present in the biomass was hydrolyzed, resulting in 14,5 ± 0,7% as $C\text{-}CO_2$ and 33,2 ± 0,5% as $C\text{-}CH_4$. The potential recovery from N and P present in the biomass was 59,2 ± 1,9% as $N\text{-}NH_4^+$ and 88,8% ± 2,3 as $P\text{-}PO_4^{-3}$, respectively. During the anaerobic digestion of mixotrophically grown microalgae, 45,5 ± 0,6% of the initial C was hydrolyzed, 13,5 ± 0,9% as $C\text{-}CO_2$ and 35,7 ± 0,5% as $C\text{-}CH_4$. The potential recovery from N and P was 69,9 ± 2,9% as $N\text{-}NH_4^+$ and 77,3 ± 1,9% as $P\text{-}PO_4^{-3}$, respectively. The energy recovery from the chemical energy fixed as biomass under photoautotrophic and mixotrophic conditions was 47,9% and 48,9%, respectively, while these values decreased to 3,3%, when referred to the total energy supplied.

Keywords: *Biomethanization, mass and energy balances, microalgae digestion, nutrients recovery, photosynthetic efficiency.*

INTRODUCTION

The current scenario of exhaustion of fossil fuel resources, increasing oil prices and global warming as a result of the accumulation of greenhouse gases in the atmosphere is strongly motivating research on biofuel production from renewable biomass. Nowadays, conventional biodiesel is mainly produced from plant oils (palm oil, canola, soybean), and despite its lower CO_2 footprint (compared to fossil fuels) it entails negative environmental impacts. Hence, land over-exploitation due the uncontrolled use of pesticides and fertilizers and the competition for cropland (which might mediate a global food crisis if expected to satisfy the current world's fuel demand) rank among the main drawbacks of conventional biodiesel.

In this context, microalgae have emerged as a promising feedstock for biofuel production based on their high photosynthetic yields, high and sustained growth rates and ability to grow in both marine, fresh and wastewaters. Besides the above mentioned advantages, microalgae have the ability to mitigate greenhouse emissions by photosynthetically fixing the CO_2 released in industrial processes and do not compete with cropland. Most of the research carried out in the last 5 years has mainly focused on microalgal biodiesel, but the high cost of axenic microalgae biomass cultivation nowadays (4-20 € kg^{-1}) (Sumi 2009, Norsker et al. 2010, Acién 2012) has limited its full scale implementation.

Anaerobic digestion appears as a promising technology for biofuel production based on its ability to use residual algal biomass as a substrate for biomethane production and its potential for recovering an important part of the nutrients (N, P) provided in the growth stage, which can offset a significant fraction of the process operating costs. In this regard, recent sustainability studies have shown that the energy input of nutrients supply constitutes the major energy cost during microalgae cultivation (Chisti 2008, Ehimen 2010, Yang et al. 2010). However, there are still significant technical–economic limitations in the cultivation and biomethanization of microalgae which restrict its full-scale implementation, among them, the lack of information about the fundamental mass and energy balances of the combined growth-digestion process.

This information is crucial to quantify the potential for energy and nutrients recovery in the overall biofuel production process.

The main objective of this work was the quantification of the C, N, and P mass balances in the integrated process of microalgae photosynthetic growth (under autotrophic and mixotrophic conditions) followed by anaerobic digestion. This study assessed on the one hand, the photosynthetic efficiency and microalgae specific growth rate at the exponential growth phase, nutrients consumption, biomass productivity and CO_2 fixation capacity of *Chlorella sorokiniana* during the growth stage, and on the other hand, the biomethane production yield and the potential for nutrients and energy recovery during anaerobic digestion. The global energy recoveries were also estimated.

MATERIALS AND METHODS

Microorganisms and growth conditions

The microalgae *C. sorokiniana 211/8k* was obtained from the Culture Collection of Algae and Protozoa of the SAMS Research Services (Argyl, Scotland) and was cultivated in SK mineral salt medium (MSM) containing (per cubic meter of distilled water): 1250 g KNO_3, 625 g $MgSO_4 \cdot 7H_2O$, 110,5 g $CaCl_2 \cdot 2H_2O$, 114,2 g H_3BO_3, 49,8 g $FeSO_4 \cdot 7H_2O$, 88,2 g $ZnSO_4 \cdot 7H_2O$, 14,4 g $MnCl_2 \cdot 4H_2O$, 7,1 g MoO_3, 15,7 g $CuSO_4 \cdot 5H_2O$, 4,9 g $Co(NO_3)_2 \cdot 6H_2O$, 500 g EDTA, 624,7 g KH_2PO_4, 1325,1 g K_2HPO_4. The pH was adjusted to 6.8 with KOH and the medium was autoclaved before use. A solution of $MgSO_4 \cdot 7H_2O$ was autoclaved separately and added to the sterile culture medium afterwards to avoid salt precipitation. Glucose, peptone and yeast extract at 3125, 62,5 and 62,5 g/m^3 were supplemented from a sterile stock solution to the SK MSM during inocula preparation. Microalgae inocula were grown in 250 ml Erlenmeyer flasks under magnetic agitation at 300 rpm in a thermostatic bath at 30 ºC under continuous illumination.

Microalgae growth

Autotrophically grown microalgae were cultivated in ten 1,25 dm^3 sterile glass bottles containing 0,5 dm^3 of a sterile Spirulina modified mineral salt medium composed of (per cubic meter of distilled water): 6805 g $NaHCO_3$, 2015 g Na_2CO_3, 78,6 g K_2HPO_4, 355,7 g NH_4Cl, 500 g K_2SO_4, 500 g NaCl, 100 g $MgSO_4 \cdot 7H_2O$, 20 g $CaCl_2 \cdot 2H_2O$, 6.8 g $FeSO_4 \cdot 7H_2O$, 42 g EDTA, 0.00025 g $ZnSO_4$, 0.0005 g $MnSO_4$, 0.0025 g H_3BO_3, 0.00025 g $Co(NO_3)_2 \cdot 6H_2O$, 0.00025 g $Na_2MoO_4 \cdot 2H_2O$, $1{,}25 \cdot 10^{-6}$ g $CuSO_4 \cdot 5H_2O$. Prior to sterilization, the bottles were flushed with He in order to establish an O_2 free atmosphere, closed with butyl septa and sealed with plastic caps.

Mixotrophically grown microalgae were cultivated in twelve 1,25 dm^3 glass bottles containing 0,5 dm^3 of a sterile Spirulina modified mineral salt medium supplemented with 1 ml of a 301,4 g/dm^3 glucose stock solution as the organic carbon source. The concentrations of carbonate and bicarbonate in the MSM were modified to represent the typical inorganic carbon concentration in domestic wastewater (inorganic carbon concentrations ranging from 100 to 150 g/m^3). Hence, MSM for mixotrophic growth was composed of (per cubic meter of distilled water): 1700 g $NaHCO_3$, 500 g Na_2CO_3, 500 g $C_6H_{12}O_6$, 78,6 g K_2HPO_4, 355,7 g NH_4Cl, 500 g K_2SO_4, 500 g NaCl, 100 g $MgSO_4 \cdot 7H_2O$, 20 g $CaCl_2 \cdot 2H_2O$, 6.8 g $FeSO_4 \cdot 7H_2O$, 42 g EDTA, 0.00025 g $ZnSO_4$, 0.0005 g $MnSO_4$, 0.0025 g H_3BO_3, 0.00025 g $Co(NO_3)_2 \cdot 6H_2O$, 0.00025 g $Na_2MoO_4 \cdot 2H_2O$, $1{,}25 \cdot 10^{-6}$ g $CuSO_4 \cdot 5H_2O$. Prior to sterilization, the bottles were flushed with He, closed with butyl septa and sealed with plastic caps. The pH of the cultivation was then decreased to 7.6 by injecting 1,6 ml of HCl (37%) and the systems were allowed to equilibrate for 2 h at 25 ºC prior to inoculation.

Once the system was balanced, the headspace concentration of CO_2 in the autotrophic system was 18 ± 0,7 %, which represented a C-CO_2 concentration of 82,7 ± 3,4 g/m^3 and 1,9 ± 0,1 % in the mixotrophic system, which represented a C-CO_2 concentration of 8,9 ± 0,5 g/m^3. The bottles were inoculated with fresh *C. sorokiniana* at an initial concentration of 11,4 ± 0,0 g/m^3 TSS (1 mL of inoculum with a concentration of 5713 g/m^3) for autotrophic growth and 11,0 ± 0,0 g/m^3 TSS (1 mL of inoculum with a concentration of 5467 g/m^3) for mixotrophic growth. The tests were incubated at 30 ºC under continuous magnetic agitation at 300 rpm and continuously illuminated at an average intensity of 82,4 ± 7,3 (µE/m^2·s) during 9,5 days for autotrophic growth and 11,5 days for mixotrophic growth. Gas samples of 100µL were taken at the beginning and end of the cultivations to record the CO_2 and O_2 headspace concentrations by GC-TCD. In addition, liquid samples of 100 ml were also drawn at the beginning and end of the growth phase to quantify the dissolved total organic carbon (TOC), dissolved inorganic carbon (IC), dissolved total nitrogen (TN = N-NH_4^+, N-NO_2^-, N-NO_3^- and $N_{organic}$), dissolved phosphorus (P-PO_4^{-3}) and microalgae biomass concentrations. The C, N and P content of the algal biomass formed was also experimentally determined.

Microalgae were harvested by centrifugation for 10 minutes at 10.000 rpm (Sorvall, LEGEND RT+ centrifuge, Thermo Scientific). The microalgae pellet was resuspended in tap water to avoid cell lysis and constituted the raw material for anaerobic digestion.

Microalgae anaerobic digestion

The anaerobic digestion of microalgae was performed batchwise in triplicate in 120 ml serum bottles filled with 80 ml of culture broth (microalgae + anaerobic inoculum), under strictly anaerobic conditions (He atmosphere) in an orbital shaker at 35 ºC. The substrate to inoculum ratio was 0.5. The inoculum used to digest the autotrophically grown microalgae was an anaerobic bacterial sludge previously adapted to microalgae for two months containing 9.8 g/dm^3 of total suspended solids (TSS) and the concentration of autotrophically grown microalgae was 13.3 g/dm^3 of TSS. On the other hand, the concentration of anaerobic inoculum and microalgae for the digestion of mixotrophically grown microalgae was 5,4 and 9,3 g/dm^3 TSS, respectively. Triplicate tests prepared as above described containing only anaerobic inoculum were used as control. The anaerobic digestion tests (74 days for biomass cultivated autotrophically and 89 days for biomass cultivated mixotrophically) were monitored by periodic measurements of the pressure of the headspace and biogas composition. Gas samples of 100µL were periodically taken to record the concentration of CO_2 and CH_4 in the biogas produced. Furthermore, liquid samples were also drawn at the beginning and end of the anaerobic digestion stage to determine the TOC, IC, TN, N-NO_2^-, N-NO_3^-, P-PO_4^{-3} and TSS concentrations. The C, N and P content of the raw algal biomass, the anaerobic inoculum and the final digested biomass was also experimentally determined.

Mass and energy balances

A mass balance calculation was conducted for each target compound evaluated (C, N and P) considering all its chemical forms at the beginning and end of both the growth and anaerobic digestion stages. The quality of the experimentation carried out was assessed by means of the recovery factor. In the particular case of the microalgae growth stage under both autotrophic and mixotrophic conditions, the recovery factor was defined as follows:

$$\text{C mass recovery (\%)} = \frac{[\text{C-CO}_2 + \text{TOC} + \text{IC} + \text{C}_{\text{biomass}}]_{\text{END POINT}}}{[\text{C-CO}_2 + \text{TOC} + \text{IC} + \text{C}_{\text{biomass}}]_{\text{START POINT}}} \cdot 100 \qquad \text{eq. [1]}$$

$$\text{N mass recovery (\%)} = \frac{[\text{N-NH}_4^+ + \text{N}_{\text{biomass}} + \text{N-NO}_2^- + \text{N-NO}_3^-]_{\text{END POINT}}}{[\text{N-NH}_4^+ + \text{N}_{\text{biomass}} + \text{N-NO}_2^- + \text{N-NO}_3^-]_{\text{START POINT}}} \cdot 100 \qquad \text{eq. [2]}$$

$$\text{P mass recovery (\%)} = \frac{[\text{P-PO}_4^{-3} + \text{P}_{\text{biomass}}]_{\text{END POINT}}}{[\text{P-PO}_4^{-3} + \text{P}_{\text{biomass}}]_{\text{START POINT}}} \cdot 100 \qquad \text{eq. [3]}$$

where the C-CO_2 is the carbon as gaseous CO_2 at the flask's headspace, TOC is the total dissolved organic carbon in the aqueous phase, IC is the dissolved inorganic carbon at the aqueous phase in equilibrium with C-CO_2 gas (CO_2 (g) ↔ CO_2 (l) + H_2O ↔ HCO_3^- + H^+ ↔ CO_3^{-2} + 2H^+), $C_{biomass}$ is the particulate carbon in the form of microalgal biomass, N-NH_4^+ is the nitrogen as ammonium, N-NO_2^- is the nitrogen as nitrite and N-NO_3^- is the nitrogen as nitrate, at the aqueous phase, $N_{biomass}$ is the particulate organic nitrogen in the biomass, P-PO_4^{-3} is the phosphorus at the aqueous phase and $P_{biomass}$ is the particulate phosphorus in the form of biomass.

The N and P balances for the anaerobic digestion stage were similar to those defined in eq [2] and eq. [3]. In the C balance, C as methane (C-CH_4) must be included:

$$\text{C mass recovery (\%)} = \frac{[\text{C-CO}_2 + \text{C-CH}_4 + \text{TOC} + \text{IC} + \text{C}_{\text{biomass}}]_{\text{END POINT}}}{[\text{C-CO}_2 + \text{C-CH}_4 + \text{TOC} + \text{IC} + \text{C}_{\text{biomass}}]_{\text{START POINT}}} \cdot 100 \qquad \text{eq. [4]}$$

Apart from evaluating the accuracy of the analytical and instrumental methods used in this study, these mass balances were used to estimate the potential for bioenergy production and nutrients recovery from the anaerobic digestion of the algal biomass, in order to recycle the nutrients hydrolyzed back to the cultivation stage.

The photosynthetic efficiency (PE) during the exponential growth phase was calculated as follows:

$$\text{PE (\%)} = \frac{\text{TCEF}}{\text{TEA}} \cdot 100 = \frac{\text{M} \cdot \text{H}}{\text{E} \cdot \text{T}} \cdot 100 \qquad \text{eq. [5]}$$

where TCEF is the total chemical energy fixed during the exponential growth phase (kJ), TEA is the total energy available for microalgae during the exponential growth phase (kJ), M is the microalgae production (g) in time duration T (d), H is the specific chemical energy content of algal biomass as heat (kJ/g) and E is the energy flow available for microalgae during the exponential growth stage (kJ/d). Under autotrophic conditions TEA represented the light energy available by microalgae, and for the mixotrophically grown microalgae, the energy provided by light and the chemical energy of glucose were considered. The specific heating value of the microalgal biomass considered in PE calculation was 21 kJ/g of microalgae (Illman 2000, Park et al. 2011).

In order to calculate E, the amount of light energy absorbed by the glass bottles was taken into account:

$$E = TE - TE \cdot G \qquad \text{eq. [6]}$$

where G is the fraction of light absorbed by the bottles glass (%) and TE is the total light energy supplied at the tests bottles during the exponential growth phase. TE was calculated by multiplying the measured light intensity (μE/m^2·s) at the top and side of the bottles by their corresponding areas and by the duration of the exponential growth phase. The overall energy recovery at the exponential growth phase was calculated based on TCEF (ER_{TCEF}) and TE (ER_{TE}) as follows:

$$(ER)_{TCEF}\ (\%) = \frac{ME}{TCEF} \cdot 100 \qquad \text{eq.[7]}$$

$$(ER)_{TE}\ (\%) = \frac{ME}{TE} \cdot 100 \qquad \text{eq. [8]}$$

where ME is the energy provided by the combustion of methane obtained during the anaerobic digestion of the biomass produced during the exponential growth phase. The heating value of the CH_4 produced during anaerobic digestion here used was 50 kJ/g of CH_4 (Perry and Green, 1999).

Under autotrophic conditions, these energy balances allowed to assess the energy recovery potential as CH_4 from the light energy captured as chemical energy in the microalgal biomass. Under mixotrophic conditions, the chemical energy of the glucose consumed was also taken into account.

Analytical procedures

TOC, IC and TN were determined using a Shimadzu TOC-5050A analyzer (Japan). $N\text{-}NO_3^-$, $N\text{-}NO_2^-$ and $P\text{-}PO_4^{-3}$ were analyzed via high-performance liquid chromatography-ion chromatography (HPLC-IC) with a Waters 515 HPLC pump (Waters, Milford, USA) coupled with an ion conductivity detector (Waters 432, Milford, USA) using an IC-Pak Anion Guard-Pak column (Waters, Milford, USA), an IC-Pak Anion HC (150 mm×4.6 mm) column (Waters, Milford, USA) and a Waters 717 plus autosampler (Waters, Milford, USA). Acetonitrile was used as eluent phase at 2 ml/min. The dissolved phosphorus and total phosphorus concentrations (total phosphorus previously digested after acidification with 18,6% HNO_3 in a microwave oven Mars Xpress, CEM, USA) were determined using a spectrophotometer U-2000 (Hitachi, Japan). All these analysis were carried out according to *Standard Methods* (Eaton et al., 2005). A Crison micropH 2002 (Crison instruments, Barcelona, Spain) was used for pH determination.

The gaseous concentrations of CO_2, O_2 and N_2 were analyzed using a gas chromatograph (Varian CP-3800, Palo Alto, CA, USA) coupled with a thermal conductivity detector and equipped with a CP-Molsieve 5A (15 m × 0.53 mm × 15 μm) and a CP-Pora BOND Q (25 m × 0.53 mm × 15 μm) columns. Injector and detector temperatures were 150ºC and 175ºC respectively. Helium was the carrier gas at 13.7 ml/min.

The light intensity was measured with LI-250 A light meter (LI-COR Biosciences, Germany) and expressed in μE/m^2·s. The photosynthetically active radiation (PAR) was assumed to have a wavelength close to 550 nm and 1 W/m^2 as PAR was calculated to be equivalent of 4.6 μE/m·s (Béchet, 2012).

Biomass concentration was estimated from culture absorbance measurements at 550 nm (OD_{550}) using a HITACHI U2000 UV/visible spectrophotometer (Hitachi Ltd., Tokyo, Japan). In addition, total suspended solids concentration was performed according to Standard Methods (Eaton et al., 2005). The determination of the $C_{biomass}$, $N_{biomass}$ and $S_{biomass}$ was performed using a LECO CHNS-932 at the Instrumental Techniques Laboratory of the Universidad Complutense de Madrid.

RESULTS AND DISCUSSION

The C, N and P mass balances were evaluated during the autotrophic and mixotrophic microalgae growth stages and the subsequent anaerobic digestion stages. All results obtained are summarized in Table 1 and Table 2, where AG and MG represent the autotrophic and mixotrophic cultures, respectively. The results are given as the average ± errors at 95% confidence interval (n = 10 for AG, n = 12 for MG and n= 3 for the anaerobic digestion tests).

	GROWTH STAGE MASS BALANCES								
	INITIAL				FINAL				
	CARBON MASS BALANCE								
	$C\text{-}CO_2$ (g/m^3)	IC (g/m^3)	TOC (g/m^3)	$C_{biomass}$ (g/m^3)	$C\text{-}CO_2$ (g/m^3)	IC (g/m^3)	TOC (g/m^3)	$C_{biomass}$ (g/m^3)	Recovery (%)
AG	82,7 ± 3,4	1020,9 ± 12,8	17,3 ± 0,1	5,3 ± 0,0	3,6 ± 0,2	931,7 ± 8,3	51,6 ± 2,7	215,8 ± 5,8	103,4 ± 0,5
MG	8,9 ± 0,5	130,8 ± 1,2	220,1 ± 3,3	4,0 ± 0,0	0,1 ± 0,0	53,8 ± 5,1	146,4 ± 3,9	167,0 ± 7,0	100,1 ± 1,9
	NITROGEN MASS BALANCE								
	$N\text{-}NH_4^+$ (g/m^3)	$N\text{-}NO_2^-$ (g/m^3)	$N\text{-}NO_3^-$ (g/m^3)	$N_{biomass}$ (g/m^3)	$N\text{-}NH_4^+$ (g/m^3)	$N\text{-}NO_2^-$ (g/m^3)	$N\text{-}NO_3^-$ (g/m^3)	$N_{biomass}$ (g/m^3)	Recovery (%)
AG	94,8 ± 0,7	3,0 ± 0,2	0,0 ± 0,0	0,9 ± 0,0	60,1 ± 0,4	3,5 ± 0,3	0,0 ± 0,0	34,5 ± 0,9	99,0 ± 0,1
MG	94,5 ± 0,5	0,0 ± 0,0	0,0 ± 0,0	0,7 ± 0,0	63,8 ± 0,5	0,0 ± 0,0	0,0 ± 0,0	30,3 ± 1,3	98,7 ±1,3
	PHOSPHORUS MASS BALANCE								
	$P\text{-}PO_4^{-3}$ (g/m^3)		$P_{biomass}$ (g/m^3)		$P\text{-}PO_4^{-3}$ (g/m^3)		$P_{biomass}$ (g/m^3)		Recovery (%)
AG	13,7 ± 0,3		0,1 ± 0,0		10,6 ± 0,4		3,3 ± 0,1		99,6 ± 3,7
MG	13,1 ± 0,2		0,1 ± 0,0		10,7 ± 0,4		2,6 ± 0,1		100,6 ± 3,9

Table. 1. C, N and P mass balances during microalgae growth under autotrophic (AG) and mixotrophic (MG) conditions.

	ANAEROBIC DIGESTION MASS BALANCES										
	INITIAL					FINAL					
	CARBON MASS BALANCE										
	$C\text{-}CO_2$ (g/m^3)	$C\text{-}CH_4$ (g/m^3)	IC (g/m^3)	TOC (g/m^3)	$C_{biomass}$ (g/m^3)	$C\text{-}CO_2$ (g/m^3)	$C\text{-}CH_4$ (g/m^3)	IC (g/m^3)	TOC (g/m^3)	$C_{biomass}$ (g/m^3)	Recovery (%)
AG	0,0 ± 0,0	0,0 ± 0,0	17,9 ± 0,0	1,2 ± 0,0	6720,6 ± 0,0	227,4 ± 11,9	520,5 ± 4,4	505,9 ± 4,9	116,7 ± 5,0	3052,1 ± 39,7	104,2 ± 1,9
MG	0,0 ± 0,0	0,0 ± 0,0	22,1 ± 0,0	0,8 ± 0,0	4459,4 ± 0,0	162,6 ± 11,1	428,5 ± 6,5	448,6 ± 20,3	214,1± 17,7	2430,9 ± 27,9	135,9 ± 0,5
	NITROGEN MASS BALANCE										
	TN (g/m^3)	$N\text{-}NO_2^-$ (g/m^3)	$N\text{-}NO_3^-$ (g/m^3)	$N_{biomass}$ (g/m^3)		TN (g/m^3)	$N\text{-}NO_2^-$ (g/m^3)	$N\text{-}NO_3^-$ (g/m^3)	$N_{biomass}$ (g/m^3)		Recovery (%)
AG	1,3 ± 0,0	0,0 ± 0,0	0,0 ± 0,0	1075,6 ± 0,0		602,2 ± 20,0	0,0 ± 0,0	0,0 ± 0,0	603,6 ± 17,7		108,7 ± 1,1
MG	1,4 ± 0,0	0,0 ± 0,0	0,0 ± 0,0	809,1 ± 0,0		567,1 ± 23,8	0,0 ± 0,0	0,0 ± 0,0	429,9 ± 20,5		123,0 ± 2,3
	PHOSPHORUS MASS BALANCE										
	$P\text{-}PO_4^{-3}$ (g/m^3)		$P_{biomass}$ (g/m^3)			$P\text{-}PO_4^{-3}$ (g/m^3)		$P_{biomass}$ (g/m^3)			Recovery (%)
AG	0,03 ± 0,0		101,7 ± 0,0			90,3 ± 2,3		11,4 ± 2,3			99,3 ± 1,2
MG	0,03 ± 0,0		69,1 ± 0,0			53,4 ± 1,3		15,7 ± 1,3			100,4 ± 1,0

Table. 2. C, N and P mass balances for the anaerobic digestion of microalgae cultivated under autotrophic (AG) and mixotrophic (MG) conditions.

Microalgae growth

The maximum microalgae production rate on a volumetric basis for autotrophic and mixotrophic exponential growth phase were 71,3 g/m^3·d and 142,3 g/m^3·d, respectively, while supplying a constant light input.

The C, N and P mass balances during photoautotrophic microalgae growth showed recovery factors of 103,4 ± 0,5%, 99,0 ± 0,1% and 99,6 ± 3,7%, respectively. This validated both the analytical and instrumental methods used in this study and the experimental protocols followed. The biomass stoichiometric formula experimentally determined for the autotrophic growth was $CH_{1,63}N_{0,14}O_{0,43}P_{0,006}S_{0,005}$, which agree well with typical microalgae formulas reported in literature. For instance, Oswald (1988), Duboc et al. (1999), Chisti (2007) and Boelee (2012) reported microalgae stoichiometric formula of, $CH_{1,7}N_{0,15}O_{0,4}P_{0,0094}$, $CH_{1,78}N_{0,12}O_{0,36}$, $CH_{1,83}N_{0,11}O_{0,48}P_{0,01}$, $CH_{1,78}N_{0,12}O_{0,36}P_{0,01}$, respectively.

Under fully photoautotrophic growth, C. *sorokiniana* was capable of assimilating 1,8 ± 0,06 g of CO_2 per g of TSS of microalgae, which agrees with the CO_2 consumed per g of TSS of microalgae reported by Lardon et al. (2009). This carbon was almost equally obtained from the C-CO_2 present in the flask's headspace (95,6% ± 0,3 of the initial C-CO_2) and the IC present in the MSM (9,4% ± 1,1 of the initial IC). The total concentration of C assimilated in the form of biomass accounted for 210,5 ± 5,8 g/m^3, with an empirical C content of 50,6%. The TOC present in the initial cultivation medium (17,3 ± 0,1 corresponding to the recalcitrant chelating agent EDTA) increased up to 51,6 ± 2,7 g/m^3 due to metabolite excretion by microalgae during their photoautotrophic growth. Based on the continuous light supply and presence of sufficient concentrations of IC (931,7 ± 8,3 g/m^3), N-NH_4^+ (60,1 ± 0,4 g/m^3) and P-PO_4^{-3} (10,6 ± 0,4 g/m^3) at the end of the cultivation stage, microalgae growth was likely limited by either the high pH values or the high O_2 concentrations present at the last stages of the cultivation process. On the one hand, at the pH of 9,1 ± 0,2 recorded at the final point of the growth stage, the carbon balance is mainly shifted toward carbonate, which represents an inorganic carbon species non-available for *C. sorokiniana* growth (Liehr 1998, de Godos et al. 2010). A high NH_3 concentrations resulting from the combination of high NH_4^+ concentrations and high pH values can uncouple the electron transport in photosystem II and compete with H_2O in the oxidation reactions leading to O_2 production (Azov and Goldman 1982). However, a potential inhibition of *C. sorokiniana* growth mediated by NH_3 with a 60,1 ± 0,4 g/m^3 of N-NH_4^+ in the aqueous phase (that implies 30,2 ± 0,4 g/m^3 of NH_3) was ruled out because no significant effect on the growth of *C. sorokiniana* was observed at 310,6 g/m^3 N-NH_4^+ at a pH of 9,5 ± 0,1 (241,2 g/m^3 N-NH_3) (de Godos et al. 2010). On the other hand, the accumulation of photosynthetic O_2 at the flask's headspace as a result of the enclosed nature of the tests could have also decreased the assimilation performance of CO_2 due to a photoxidative damage of the microalgal cells (24,5 ± 1,1 g/m^3 of O_2 in the aqueous phase) and the potential competition between O_2 and CO_2 for reaction with the enzyme ribulose bisphosphate carboxylase (RubisCO), which is a key catalyst of the Calvin cycle to transform CO_2 to organic compounds (Tortell 2000, Galmés et al. 2005, Madigan et al. 2009).

TN concentration, initially in the form of N-NH_4^+, decreased from 94,8 ± 0,7 g/m^3 to 60,1 ± 0,4 g/m^3, concomitantly with an assimilation of 33,6 ± 0.9 g/m^3 in the form of $N_{biomass}$. Neither NO_2^- nor NO_3^- were produced by microalgae in significant amounts. The empirical N content of the biomass was 8,1% of the TSS of microalgae. The concentration of P-PO_4^{-3} consumed during photoautotrophic growth (3,1 ± 0.6 g/m^3) correlated with the concentration of $P_{biomass}$ produced (3,2 ± 0.1 g/m^3), with an empirical microalgae P content of 0,8%. In this context, the phosphorus content of algal cells can vary from 0,2% to 3,9% under different cultivation conditions (Powell et al., 2009). This wide range is due to the occurrence of a luxury uptake of phosphorus by some microalgae species, which represents the storage of phosphorus within the biomass in the form of polyphosphate over structural phosphorus (Powell et al., 2008).

The C, N and P mass balances during mixotrophic microalgae growth resulted in recovery factors of 100,1 ± 1,9%, 98,7 ±1,3% and 100,6 ± 3,9%, respectively.

The biomass stoichiometric formula experimentally determined for the mixotrophic growth was $CH_{1,68}N_{0,16}O_{0,48}P_{0,006}S_{0,008}$. Under these conditions, the microalgae were able to assimilate 44,1 ± 2,5 mg of the initial IC (IC + C-CO_2), which represents 64,6 ± 2,7% of the total $C_{biomass}$ formed in the cultivation system. The quantity of C-CO_2 removed during mixotrophic growth was 99,3 ± 0,4% of the initial C-CO_2 at the flask's headspace with a pH of 9,2 reported at the end of the growth stage.

Despite *C. sorokiniana* reached higher final biomass concentrations under autotrophic growth, *C. sorokiniana* productivity during the exponential growth phase was higher under mixotrophic conditions due to the absence of the initial lag phase recorded during autotrophic growth (Figure 1). In this regard, microalgae use the enzyme carbonic anhydrase, located on the surface of the cell, to promote the conversion of dissolved CO_2 to HCO_3^-, the latter being transported into the cell and reversed to CO_2 by the cytoplasmatic carbonic anhydrase close to the RubisCO catalytic sites (Chen et al. 2009).

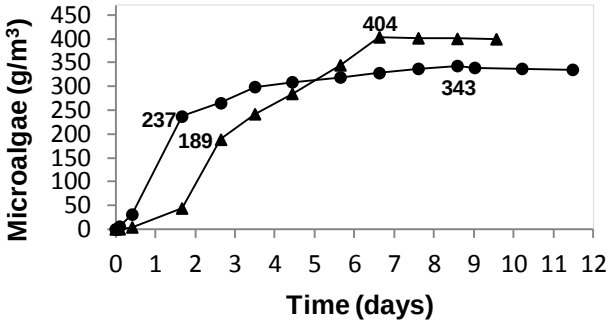

Figure 1. Microalgae growth evolution under autotrophic (▲) and mixotrophic conditions (●) in the modified *Spirulina* MSM.

Due to the high concentration of CO_2 in the flask headspace (18%) at the beginning of the autotrophic growth tests, the photosynthetic efficiency decreased and therefore a longer lag phase was recorded compared to mixotrophic conditions (1,9% of CO_2 in the flask's headspace), which corresponded to an specific growth rate at the exponential growth phase (μ) of 1,5 d^{-1}, compared to 1,6 d^{-1} for mixotrophic growth. However, the mixotrophic growth rate decreased drastically at the end of the exponential growth phased and the concentration of biomass remained practically constant with a maximum biomass concentration of 343 g/m^3.

In this context, only 0,17 ± 0,0 g of C-glucose per g of TSS of microalgae were assimilated in presence of glucose in the Spirulina modified MSM, which represented 13,8 ± 1,2% of the initial C-glucose in the MSM (203 ± 3,3 g/m^3) and 35,4 ± 4,1% of the total $C_{biomass}$ produced (163,0 ± 7,0 g/m^3 with an empirical C content of 48,0%).

In addition, in order to explain the low organic carbon assimilation rates recorded under mixotrophic conditions, an experiment with 310 g/m^3 of C-glucose was carried out in modified *Spirulina* MSM and SK MSM to compare the microalgae growth during the exponential growth phase (Figure 2).
The results here obtained clearly show a partial glucose mediated inhibition in Spirulina MSM in contrast to SK MSM. The reasons underlying this inhibition are unknown and deserve further investigation.

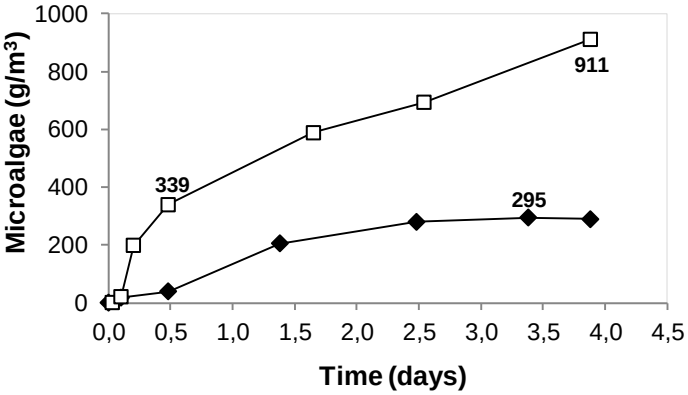

Figure 2. Microalgae growth evolution under mixotrophic conditions with 310 g/m^3 of C-glucose in Spirulina mineral salt medium (♦) and in SK mineral salt medium (□).

The concentration of TN, in the form of N-NH_4^+, decreased from 94,5 ± 0,5 g/m^3 to 63,8 ± 0,5 g/m^3, with an assimilation of 29,6 ± 1,3 g/m^3 in the form of $N_{biomass}$. Neither NO_2^- nor NO_3^- were produced by microalgae. The empirical N content of the biomass was 8,7% of the TSS of microalgae. The P-PO_4^{-3} concentration consumed during mixotrophic growth (2,4 ± 0.3 g/m^3) correlated with the concentration of $P_{biomass}$ produced (2,5 ± 0.1 g/m^3), with an empirical microalgae P content of 0,7%.

Anaerobic digestion mass balances

The C, N and P mass balances of the anaerobic digestion of the microalgae cultivated under autotrophic conditions showed recovery factors of 104,2 ± 1,9%, 108,7% ± 1,1% and 99,3 ± 1,2%, respectively.

Microalgal particulate carbon represented 99,7 ± 0,0% of the initial carbon in the system at the beginning of the anaerobic digestion (Figure 3), while dissolved inorganic carbon accounted for the remaining carbon (0,3 ± 0,0%).

$C_{biomass}$ concentration decreased from 6720,6 ± 0,0 g/m^3 to 3052,1 ± 39,7 g/m^3, which represents an hydrolysis of 54,6% ± 0,6 of the initial particulate carbon. During the anaerobic digestion, 227,4 ± 11,9 g/m^3 of C-CO_2 and 520,5 ± 4,4 g/m^3 of C-CH_4 were produced. This carbon speciation entails that approximately, 87,3 ± 1,8% of the hydrolyzed $C_{biomass}$ was converted to biogas (47,7 ± 0,5% of the initial carbon speciated in 14,5 ± 0,7% as C-CO_2 and 33,2 ± 0,5% as C-CH_4).

The remaining hydrolyzed carbon was not transformed into biogas but present in the systems as dissolved inorganic carbon (7,2 ± 0,1%). The amount of dissolved organic carbon at the end of the anaerobic degradation however negligible (1.7 ± 0,1%).

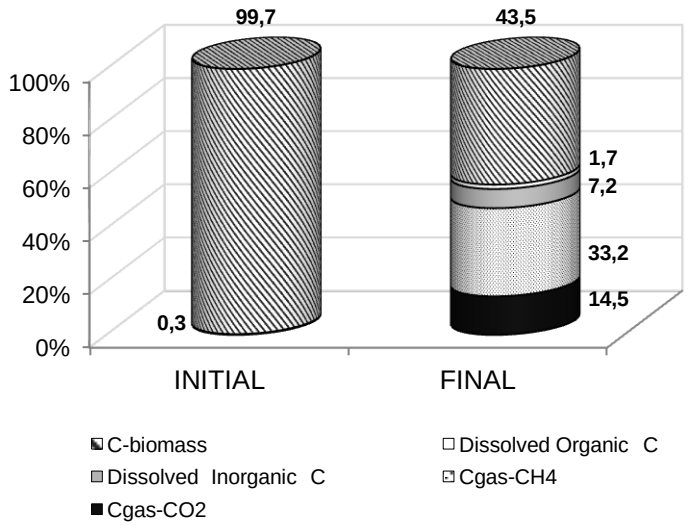

Figure 3. Carbon distribution during anaerobic digestion of microalgae cultivated under autotrophic conditions.

Approximately 59,2 ± 1,9% of the initial particulate organic nitrogen ($N_{biomass}$) was released to the aqueous phase as N-NH_4^+. The potential recovery of the particulate phosphorous ($P_{biomass}$) at the initial point was 88,8% ± 2,3. A possible explanation for this high recovery of P-PO_4^{-3} (higher than the corresponding structural P released from microalgae hydrolysis) might be the release during the anaerobic digestion of polyphosphates accumulated in microalgal cells at the previous growth stage. In this context, Powell et al. (2008, 2009) observed that the accumulation and subsequent utilization of two types of polyphosphate acids presents in microalgae (acid-soluble polyphosphate (ASP) and acid-insoluble polyphosphate (AISP)) is a function of the culture medium phosphate concentration. AISP is believed to be a form of phosphorus storage that is not utilized when the microalgae is not phosphate-starved while ASP is involved in microalgae metabolism and can act as a short term form for phosphorus storage. Hence, microalgae, like phosphate accumulating microoorganisms (PAO) (Sathasivan, 2009, Shyam, 2008) might accumulate phosphorus above its structural P requirements during aerobic growth, which could be further released under anaerobic conditions.

The C, N and P balances of the anaerobic digestion of microalgae cultivated under mixotrophic conditions resulted in recovery factors of 135,9 ± 0,5%, 123,0 ± 2,3% and 100,4 ± 1,0% respectively. A possible explanation for the high C and N recovery factors here recorded might be the low initial activity of the anaerobic sludge used. In this context, the amount of microalgae hydrolyzed, and consequently, the quantity of biogas produced and N released to the aqueous phase might have been overestimated (since it is calculated as the difference between the algal tests and the control test). As above explained, the release of P was not directly proportional to the hydrolysis of biomass due to the accumulation of phosphorus above microalgae structural P requirements.

Microalgal particulate carbon represented 99,5% of the initial carbon in the system at the beginning of the anaerobic digestion, while dissolved inorganic carbon accounted for the remaining carbon (0,5%).
The concentration of $C_{biomass}$ decreased from 4459,4 ± 0,0 g/m^3 to 2430,9 ± 27,9 g/m^3, which represents an hydrolysis of 45,5 ± 0,6% of the initial particulate carbon. During anaerobic digestion, 162,6 ± 11,1 g/m^3 of C-CO_2 and 428,5 ± 6,5 g/m^3 of C-CH_4 were formed, which represented 49,2 ± 0,5% of the initial carbon (13,5 ± 0,9% as C-CO_2 and 35,7 ± 0,5% as C-CH_4).
The remaining hydrolyzed carbon not transformed into biogas was present in the system as dissolved inorganic carbon (7,4% ± 0,3) and dissolved organic carbon (3.5% ± 0,3).

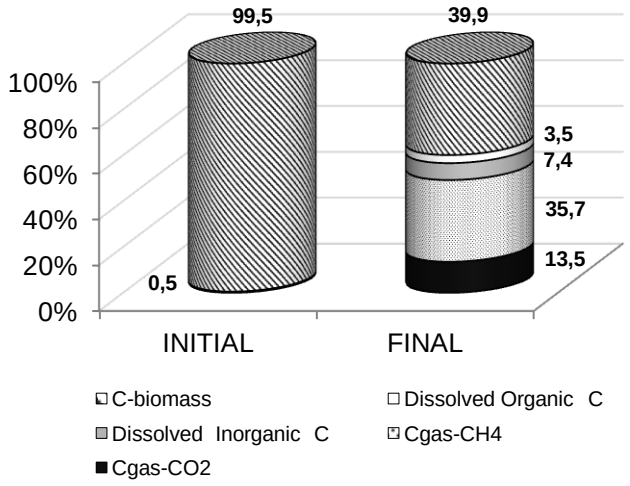

Figure 4. Carbon distribution during anaerobic digestion of microalgae cultivated under mixotrophic conditions.

Approximately 69,9 ± 2,9% of the initial particulate nitrogen ($N_{biomass}$) was released as N-NH_4^+ to the aqueous phase. The potential for particulate phosphorous ($P_{biomass}$) recovery was also high (77,3 ± 1,9% of the initial particulate P).

Energy balances

Microalgal photosynthesis released 1,0 ± 0.0 g of O_2 per g of CO_2 consumed, which agrees with the theoretical oxygen production estimated by Barh et al. (2011). The overall PE recorded during the exponential growth phase of *C. sorokiniana* under photoautotrophic and mixotrophic conditions was 4,6% and 5,0%, respectively, with a maximum PE reached of 7,6% for the autotrophic growth and 7,4 for the mixotrophic growth. These values agree with the typical PEs reported in outdoors tubular photobioreactors, which range from 3,6 to 6,3% (Acién, 2012, Béchet et al., 2012).

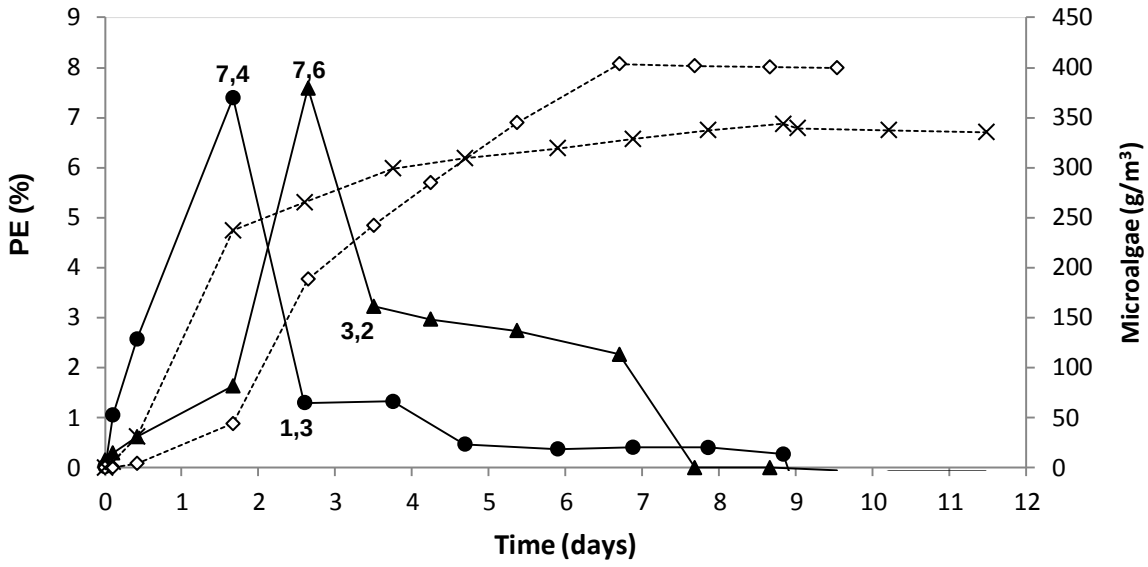

Figure 5. Time course of % PE and microalgae growth under autotrophic conditions (▲/ solid line) and (◊ / dotted line) respectively, and time course of % PE and microalgae growth under mixotrophic conditions (● / solid line) and (x / dotted line), respectively.

A global energy balance considering both microalgae growth and anaerobic digestion was conducted. Hence, the amount of energy fixed during microalgae growth was compared with the potential energy obtained from the CH_4 produced during its further anaerobic digestion.

	AG	MG
Total chemical energy fixed TCEF (kJ) *	1,5	2,2
Total energy supplied TE (kJ) *	22,0	31,9
Methane energy ME (kJ) *	0,7	1,1
Energy recovery $(ER)_{TCEF}$ (%) *	47,9	48,9
Energy recovery $(ER)_{TE}$ (%) *	3,3	3,3

Table 3. Global energy balances of integrated process: microalgae photosynthetic growth followed by anaerobic digestion.
** During the exponential growth phase.*

TCEF was thus directly proportional to the biomass produced during microalgae growth. Due to the high productivity of biomass during the exponential growth phase under mixotrophic conditions, the TCEF was higher than under phototrophic growth. The energy recovery from the TCEF was similar under photoautotrophic and mixotrophic conditions, with 47,9% and 48,9% energy recoveries, respectively. Despite the TE during mixotrophic growth was higher compared to autotrophic growth (energy available as glucose was taken into account), the methane obtained during the anaerobic digestion of the biomass produced during the mixotrophic exponential growth phase was higher compared to autotrophic growth. As a result, the global energy recovery based on the TE showing a energy recoveries of 3,3% under phototrophic and mixotrophic conditions.

CONCLUSSIONS

The recovery factors obtained in the C, N and P mass balances (≈ 100 %) validated the analytical and instrumental methods used in this study and the experimental protocols followed. Under autotrophic and mixotrophic growth, the biomass stoichiometric formula were $CH_{1,63}N_{0,14}O_{0,43}P_{0,006}S_{0,005}$ and $CH_{1,68}N_{0,16}O_{0,48}P_{0,006}S_{0,008}$, respectively, which confirmed that the carbon source ($C\text{-}CO_2$ and C-glucose) did not significantly modify the microalgae composition. *C. sorokiniana* showed a similar specific growth rate at the exponential growth phase under autotrophic and mixotrophic conditions, which involved a maximum PE reached of 7,6% and 7,4 %, respectively. However, the maximum microalgae production rate recorded during the exponential autotrophic growth phase was 71,3 $g/m^3 \cdot d$ compared to 142,3 $g/m^3 \cdot d$ under mixotrophic conditions due to the longer lag phase under autotrophic growth. The results obtained during the anaerobic digestion of microalgae cultivated autotrophically and mixotrophically showed that about 50% of the initial $C_{biomass}$ was hydrolyzed, resulting in 14% as $C\text{-}CO_2$ and 35% as $C\text{-}CH_4$. The hydrolyzed carbon was not transformed into biogas remained in the systems as dissolved inorganic carbon. The potential recoveries of $N_{biomass}$ and $P_{biomass}$ under autotrophic and mixotrophic conditions were ≈ 65% as $N\text{-}NH_4^+$ and 83% as $P\text{-}PO_4^{-3}$, respectively, which implies a significant reduction in the operating costs of the microalgae cultivation process, because nearly 45% of all fossil energy input during microalgae cultivation is linked to fertilizers supply. The energy recovery as methane during the anaerobic digestion from the chemical energy fixed by biomass under photoautotrophic and mixotrophic growth was ≈ 48%, while these values decreased to ≈ 3%, when referred to the total energy supplied.

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