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From waste to energy: Microalgae production in wastewater and glycerol

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HIGHLIGHTS

- Highest biomass and lipid productivities where achieved at mixotrophic cultivation.
- Lipids from mixotrophic biomass were more suitable for biodiesel production.
- Nutrient removal achieved below the most strict threshold limits.
- Positive energy production scenarios can be designed coupling distinct processes.

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ABSTRACT

The present work aimed to evaluate the auto/mixotrophic growth of microalgae using domestic wastewater (WW) amended with glycerol aiming biofuels production. The best results were obtained with the highest glycerol supplementation (50 mM). In such condition, *Chlorella vulgaris* and *Botryococcus terribilis* showed a biomass productivity of 118 and 282 mg l^{-1} d^{-1}, which produced about 18 and 35 mg l^{-1} d^{-1} of lipids, respectively. Thus, if scaled-up (200 m^3 d^{-1} of WW, 240 working days y^{-1}) biomass and lipid yields may be about 5.6 tons y^{-1} and 894.2 kg y^{-1} or 13.5 tons y^{-1} and 1.6 tons y^{-1} for *C. vulgaris* and *B. terribilis*, respectively. The mixotrophic production of lipids can generate high quality biodiesel according to estimations using their fatty acids profiles. The whole process can be advantageously combined with the production of other biofuels (e.g. methane and bio-ethanol) in a biorefinery scenario. This combination of algal biomass production with waste treatment (WW amended with glycerol) can have a significant impact in the water treatment sector and local markets.

1. Introduction

It is estimated that the volume of domestic effluents generated by North America, Europe and Latin America is of approximately 70, 63 and 47 km^3 y^{-1}, respectively [1]. Thus, the current main challenge of a Wastewater Treatment Plant (WWTP) is not only to produce reusable clean water, but it is also to find new technologies for supporting such an activity [2]. Conventional techniques can remove only a fraction of the total nitrogen (40%) and phosphorous (12%) present in the effluent. In order to improve the process new methods (tertiary steps) and, consequently, additional costs are required [3]. The European Directive 98/15/EC establishes a threshold of 10 and 1 mg per liter for total N and P. WWTP effluents commonly show N and P values around 20–70 and 4–12, respectively [3]. Therefore, there is still a clear need for new developments and biological systems are often considered to be the ideal means for responding to such a demand [4]. The economic costs are, however, a primary concern once improved nutrients removal would require an overall increase in energy consumption of about 60–80% [5]. Therefore, in order to reduce costs, new systems should explore the combination of wastewater treatment with the production of renewable energy in order to offset final costs [6,7].

Microalgae based systems have shown a high potential to assist with nutrient removal [8–10]. On the other hand, such a process can be improved if treatment is associated with generation of valuable co-products [7]. Biofuels have been advocated as a suitable option to replace fossil fuels [11,12]. However, several social and environmental issues are associated with increasing land crops

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based biodiesel and microalgae based systems were identified as capable of overcoming both economic and ecological problems [12–14]. In addition, microalgae systems can generate further commodities such as bio-ethanol, bio-kerosene, bio-plastics, biohydrogen, biogas, and other chemicals derivatives [15–17].

Although microalgae systems show high potential, the main bottleneck toward an effective application in the energy sector is the cost associated with both upstream and downstream processing [45,12]. In complement to the commonly explored autotrophic activity, mixotrophic algae systems have also been considered as a viable alternative for supporting innovative bioprocesses [18,19,11,10]. Mixotrophic based systems have been previously positively tested for *Chlorella* and *Botryococcus* [20–23]. These microalgae genera have been shown to have a high potential for the production of bioenergy and they also have been used for wastewater treatment. However, since the metabolic response may be strain specific, it is still necessary to evaluate their performance and, most importantly, assess their potential lipid yields at such condition.

Finding cheap supplemental carbon sources for algae cultivation is important for minimizing the economic impact. Glycerol, for instance, is currently being produced at significant amounts as the by-product of biodiesel transesterification [24] and, in 2010; the worldwide production was of about 1.8 billion liters [25] with a commercial demand of only 0.8 million per year [24]. Thus, currently, glycerol is cheaply available and with very poor commercial perspectives. Some algae mixotrophic systems have been reported using glycerol as carbon source [11,23,26], but their results regarding biomass and lipid yields are in need of complementation.

Therefore, the use of glycerol as organic supplementation for algal growth is an innovative suggestion and it may significantly contribute to environmental and economic advantages for WWTP worldwide. Thus, the aim of the present work was to evaluate the potential of using glycerol as carbon supplementation to WWTP effluent and its effects on supporting the mixotrophic growth of lipid producing microalgae.

2. Materials and methods

2.1. Microorganism and culture conditions

Chlorella vulgaris Beijerick 1890 (IBL-C105) and *B. terribilis* Komaréck *1990* (IBL-C115), which were kept on the Microalgae Bank [13] of the Marine Biology Lab (LABIOMAR) of Federal University of Bahia, Brazil, were prepared using sterilized CHU-13 medium [27]. Trials were operated in 1-l Erlenmeyer flasks. Seed concentration of 0.2 g l^{-1} was used for both strains and operational conditions were as follows: constant shaking (90 bpm), aeration (with 2.5% CO_2 supplementation), photoperiod of 12:12 light/dark cycles, luminance of 174 μE/m^2/s and incubation at constant temperature of 25 ± 1 °C.

2.2. Experimental set-up

The domestic effluent was collected after the pretreatment stage (physical removal of large particles and fat materials) at the "Moriçoca" Wastewater Treatment Plant (WWTP) Salvador, Bahia, Brazil. Glycerol ($C_3H_8O_3$) was purchased from Synth®. The experiments were carried out in triplicates. Glycerol was tested in the following concentrations: 6.25 mM, 12.5 mM, 25.0 mM and 50 mM. Higher concentrations were not tested once it has been observed an increase of over two-folds in the viscosity of the medium (data not shown). Such an effect affected negatively the cultivation. Such increase made both growth and harvesting difficult and would mean the inclusion of a confusing variable in the test.

2.3. Analytical methods

Microalgae growth was daily monitored by optical density (OD_{680nm}), pH, temperature (°C), turbidity (NTU) and total suspended solids (g/L) [32]. Biomass was recovered by centrifugation (4500 g) followed by freezing and lyophilization. Algal biomass was also analyzed for (% dry weight): carbohydrates [28], total lipids [29], chlorophyll α [18], total carotenoids [18] and protein contents [30]

Fatty-acids profile was determined by the capillary column gas chromatographic method applied to the oil methyl esters [31]. The amount of total fatty acids was obtained by transesterification into the corresponding methyl esters (FAME), through saponification with NaOH in methanol, followed by methylation with BF3 catalyst (12% in methanol). The FA methyl esters (FAME) were extracted with iso-octane and stored in an inert atmosphere (N_2) in freezer at −18 °C. The FAME separation was performed on a gas chromatograph (Varian® 3800) equipped with a flame ionization detector (GC-FID) and a fused silica capillary column Elite-WAX (30 m × 0.32 mm × 0.25 mm). The analysis parameters were as described by [33]. The injections were performed in duplicate for each extraction in volume of 1 μl. FAME's were identified by comparing their retention times with those of authentic standards (189-19, Sigma–Aldrich®, USA). The quantification of fatty acids, expressed in mg g^{-1} of the samples, was performed with internal standard (C23:0 Sigma®, USA). Samples of the wastewater effluents were analyzed (Table 1) for ammonium (N-NH_4), nitrate (N-NO_3), nitrite (N-NO_2), total nitrogen (TN), phosphate (P-PO_4), chemical oxygen demand (COD), pH, conductivity, turbidity and total suspended solids (TSS) according to [32]. Viscosity was assessed by the method of Stokes [33].

2.4. Data analysis

Kinetic parameters were estimated using a sigmoid model (Sigma Plot® v.12) as described by Chinalia and Killham [34]. The software is equipped with a statistical package for testing the fitness of the model in describing the biological response. The results are expressed as probability ($p < 0.05$). The kinetic parameters were also crosschecked using linear regression of the exponential phases. Such an approach was applied on the experimental data and their Ln transformed values, respectively.

A parametric analysis of variance (ANOVA) was carried out in order to assess the differences among tested groups. As post hoc test, it was used the Tukey's multiple comparison test (MCT). All analysis were carried out using Graph Pad Instat® software (v. 3, 2008), at the significance level of 5% ($p < 0.05$). Linear regression approach was carried out to assess the correlation between variables (with at least 5 points, R^2 results are shown in the text).

The year round productivities were calculated based on the results obtained in this research which were scaled-up in order to contemplate a full-scale scenario. Further details of this approach are described by [35,36]. The nutrient removal rates were calculated considering the total amounts removed during the incubation period (days). Therefore results are expressed as mg of nutrient removed per day. The assessment of biodiesel quality was carried out based on algal FAMEs profiling as described by Nascimento et al. [13].

Table 1
Chemical composition of wastewater (WW) and in all experimental conditions (glycerol addition) for both strains at the beginning of the trials.

Parameters	*Chlorella vulgaris*						*Botryococcus terribilis*					
	WW	WW	WW + 6.1 mM	WW + 12.5 mM	WW + 25 mM	WW + 50 mM	WW	WW + 6.1111 M	WW + 12.5 mM	WW + 25 mM	WW + 50 mM
Turbidity (NTU)	280	265	225	260	260	220	205	240	215	200	195
Salinity (‰)	0	–	–	–	–	–	–	–	–	–	–
Viscosity (μ kg m s)	0.023	0.021	0.017	0.021	0.019	0.025	0.02	0.021	0.02	0.021	0.023
pH	6.91	7.19	7205	7.2	7165	7165	7245	7205	7205	7.14	7185
COND (μS/cm)	808	–	–	–	–	–	–	–	–	–	–
Suspended Solids (g SS L^{-1})	0.2	0.41	0.38	0.4	0.4	0.38	0.42	0.5	0.46	0.49	0.43
COD (mg O_2L^{-1})	1000.1	1002.3	1012.9	2025.9	3750.8	6195.6	995.63	1095.11	2190.22	3850	6078.9
Total Nitrogen (mg Nt L^{-1})	52.03	43.67	48.63	47.56	49.54	46.78	51.23	52.41	50.39	48.86	50.02
NH_4 (mgN-NH_4 L^{-1})	43.85	39.44	44.02	43.85	44.19	42.83	47.42	46.06	45.89	44.53	45.72
NO_3 (mgN-NO_3 L^{-1})	3.06	2.47	1.91	2.06	1.93	2.2	3.18	2.41	2.01	2.56	2.64
NO2 (mgN-NO_2 L^{-1})	0.13	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
PO4 (mgP-PO_4 L^{-1})	7.11	6.66	9.32	9.17	9.37	9.79	11.5	11.41	10.84	11.59	10.56

3. Results and discussion

3.1. Biomass production

Growth kinetic parameters for *C. vulgaris* on wastewater effluent and with distinct glycerol supplementations are shown in Figure 1A and Table 2. Higher values of biomass productivity were observed in the trials with 25 and 50 mM of glycerol (107 and 118 mg l^{-1} d^{-1}). Liang et al. [21] reported that the glycerol concentration of 100 mM increased *C. vulgaris* biomass productivity in 10-folds (from 10 to 102 mg l^{-1} d^{-1}). The results from the present research, however, showed that glycerol at lower concentrations (6.25 and 12.5 mM) affected negatively algal productivity when compared to the control (54.2 and 46.4 mg l^{-1} d^{-1}, respectively). Low glycerol concentrations (12.5 mM) may trigger mixotrophic metabolism, but it is not enough for supporting an enhanced production of algal biomass. On the other hand, productivity was higher at glycerol concentrations of 25 and 50 mM.

Growth kinetics parameters for *B. terribilis* on wastewater amended with glycerol are shown in Figure 1B and Table 2. Different from *C. vulgaris*, the ability of this former species to support biological processes has not yet been comprehensively tested. Similarly to *C. vulgaris*, growth was negatively affected at low glycerol concentrations. Recently, Nascimento et al. [13] have reported relatively higher biomass productivity grown autotrophically (200 mg l^{-1} d^{-1}). In the present study, *B. terribilis* showed a biomass productivity of about 224 and 282 mg l^{-1} d^{-1} with wastewa-

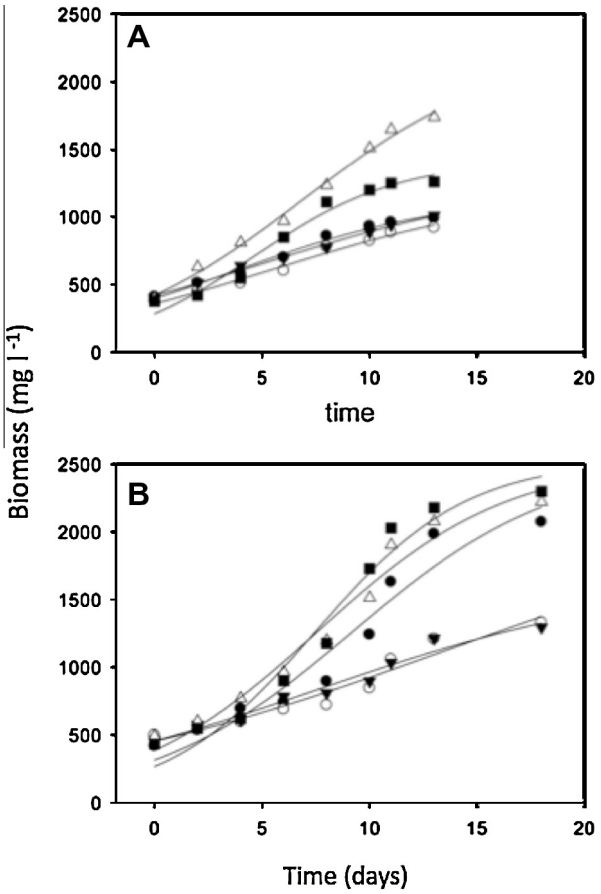

Fig. 1. Growth curves of the strains at all tested conditions: *Chlorella vulgaris* in wastewater (WW) ▼, with glycerol addition of 6.1 mM ○, 12.5 mM ●, 25 mM △ and 50 mM ■ (A) and *Botryococcus terribilis* in wastewater (WW) ●, with glycerol addition of 6.1 mM ○, 12.5 mM ▼, 25 mM △ and 50 mM ■ (B).

Table 2
Kinetic parameters, nutrient removal rates and lipid productivity of both *C. vulgaris* and *B. terribilis* strains in all tested conditions. All kinetic parameters are originated from 3 biological replicates.

Parameters	*Chlorella vulgaris*					*Botryococcus terribilis*				
	WW	WW + 6.1 mM	WW + 12.5 mM	WW + 25 mM	WW + 50 mM	WW	WW + 6.1 mM	WW + 12.5 mM	WW + 25 mM	WW + 50 mM
X_o (mg SS L^{-1})	0.41	0.38	0.4	0.4	0.38	0.42	0.50	0.46	0.49	0.43
X_M (mg SS L^{-1})	1.01	1.31	1.77	1.00	0.94	2.17	2.37	2.28	1.31	1.39
Biomass productivity (mg l^{-1} d^{-1})	57.7	54.25	46.48	107.69	118.45	224.81	102.42	103.43	184.27	282.86
Lipid productivity (mg l^{-1} d^{-1})	15.74	11.8	11.28	14.43	18.63	56.2	9.78	26.89	30.68	35.07
μ (d^{-1})	0.08	0.08	0.08	0.13	0.11	0.11	0.06	0.06	0.1	0.11
R_2	0.99	0.97	0.99	0.99	0.99	0.99	0.98	0.99	0.97	0.99
Exponential phase	6	6	8	10	6	5	5	3	5	3
Culture days	13	13	13	13	13	18	18	18	18	18
COD (mg l^{-1} d^{-1})	75.56	56.88	114.85	271.79	448.47	53.71	50.80	96.86	204.26	320.83
NT (mg l^{-1} d^{-1})	3.21	3.55	3.47	3.52	3.39	2.74	2.77	2.65	2.54	2.62
NH_4 (mg l^{-1} d^{-1})	3.03	3.38	3.37	3.40	3.29	2.63	2.56	2.55	2.47	2.54
NO_3 (mg l^{-1} d^{-1})	0.17	0.13	0.14	0.13	0.15	0.16	0.11	0.10	0.12	0.11
PO_4 (mg l^{-1} d^{-1})	0.51	0.72	0.71	0.72	0.75	0.40	0.63	0.60	0.64	0.35

Abbreviations: X_o = initial biomass concentration; X_m = final biomass concentration; COD = Chemical oxygen demand; Nt = total nitrogen; NH_4 = ammonium nitrogen; NO_3 = nitrate nitrogen; PO_4 = phosphate phosphorus.

ter effluent (WW) supplemented with glycerol (WW + 50 mM). Biomass production of the *Botryococcus* genus is among the highest reported in the literature [38]. It has been shown that *Botryococcus* biomass productivity may vary from 19 to 195 mg l^{-1} d^{-1} under autotrophic or heterotrophic conditions [39,20,39]. In the present work, biomass production of *B. terribilis* was over 200 mg l^{-1} d^{-1} in either autotrophic or mixotrophic conditions supplemented with 50 mM glycerol.

3.2. Nutrient removal

COD removal ratios were higher than 70% (Table 2). There is clear linear correlation between glycerol concentrations and COD removal rates (R^2 = 0.98), a product of the mixotrophic growth. Similar COD removal ratios have been observed previously [35,40,41]. However, the values reported by this research are significantly higher than what has been found elsewhere [42,43]. It is worth noticing that COD removal rates at high glycerol concentrations reached efficiencies of about 73% at a starting point of 3500 and 6000 mg l^{-1} O_2 (Table 2). In some cases, COD effluent values generated by the mixotrophic system (25 and 50 mM) were above the legal thresholds regulated by the European Directive. The common WWTP response in such cases is to recirculate the effluent through the aerobic treatment stage. It should be highlighted, however, that the supplementation of glycerol has also increased COD removal rates and the production of a valuable product, oil-rich algae biomass. In addition, it was observed almost complete removal of N and P. Therefore the gains with this process are very advantageous.

B. terribilis and *C. vulgaris* showed very similar nitrogen removal rates, about 3.4 mg l^{-1} d^{-1}, with final concentrations below 10 mg l^{-1} (Table 2). This value corresponds to the accepted threshold regulated by the European directive 98/15/EC. Phosphorus is rarely a limiting factor when wastewater effluents are used for cultivating algae [4]. Phosphorus removal rates by *C. vulgaris* varied from 0.5 to 0.7 mg l^{-1} d^{-1}. *B. terribilis* showed a lower efficiency in removing phosphorus when compared to *C. vulgaris* (Table 2). The aim is to reach close the target of 1 mg l^{-1}, which is the threshold of the cited European directive. At higher glycerol concentrations (12.5–50 mM) phosphorus removal rate was of 0.6 mg l^{-1} d^{-1}, approximately. This is very promising to support strategies for phosphorus removal at tertiary treatment stages. In addition, such a process can also be useful for recovering P [44].

3.3. Biorefinery possibilities

Apart from lipids and proteins, full-scale systems based on microalgae can produce other valuable substances [12]. Often, these substances can be produced simultaneously and recovered separately during the production line. In this context several authors suggested an integrating concept which has been defined as algal biorefinery systems [12,45]. Such strategy would allow a more efficient exploitation of microalgae-based systems. The main chemical fractions that support the refinery of the algal biomass can be found in Table 3. Both strains have shown high protein content (60–70%), but their production did not correlate with increasing glycerol concentrations (R^2 of 0.34 and 0.00 for *Chlorella* and *Botryococcus*, respectively). *C. vulgaris* showed a significant correlation between glycerol removal rates and the carbohydrates contents (R^2 = 0.80). Despite the fact that such response was not observed with *Botryococcus terribillis* (R^2 = 0.00), the total amount of carbohydrates where higher for the former than the values observed for the latter (Table 3).

If algal carbohydrates were to be used for ethanol production, for instance, 5.6 and 13.5 tons of algal biomass y^{-1} could generate 196 and 506 l of ethanol, for *Chlorella* and *Botryococcus*, respec-

Table 3
Biochemical composition of the microalgae strains as percentage of dry weight (%) and concentration in dry weight (μg/mg).

Strain/condition	Biomass composition				
	Carbohydrates (%)	Total lipids (%)	Protein (%)	Chlorophyll α (μg/mg)	Total carotenoids (μg/mg)
C. vulgaris					
WW	3.9a	27.3a	65.0a	4.71a	1.81a
WW + 6.12 mM	5.2ab	21.7b	70.0b	2.53b	0.86b
WW + 12.5 mM	5.9ab	24.3a	69.0c	2.25b	0.84b
WW + 25.0 mM	6.8b	13.4c	69.0c	0.20c	0.04c
WW + 50.0 mM	7.3b	15.7c	70.0b	0.25c	0.04c
B. terribilis					
WW	7.8a	25.0a	67.0a	3.76a	0.84a
WW + 6.12 mM	7.lab	9.5b	73.0b	2.66b	0.74b
WW + 12.5 mM	6.2ab	26.0a	67.0a	1.38c	0.52c
WW + 25.0 mM	4.9b	16.7c	75.0c	0.36d	0.05d
WW + 50.0 mM	7.9a	12.4d	68.0a	0.19e	0.00d

Different letters show statistical significance ($p < 0.05$) by ANOVA test. Test carried out to compare each biochemical fraction, among the treatments within each strain. Data showed are the mean of two replicates. Standard deviations not present because were lower than 10% for all treatments.

tively (table 5). The algal biomass waste, which is commonly produced during the process of lipid extraction for biodiesel production, can also be used for the co-generation of methane. Table 5 shows that the potential year round production of methane may vary between 1346 and 3258 $m^3\ y^{-1}$, for *Chlorella* and *Botryococcus*, respectively. It was also observed that glycerol supplementation decreased pigment production for favoring mixotrophic growth. This observation correlates with the obtained values for chlorophyll and carotenoids (Table 3).

In this experiment, the accumulation of lipids by *C. vulgaris* varied from 13% to 27% of dry weight; and the respective range shown by *B. terribilis* are of 9.5–25% (Table 3). Although higher lipid contents may have been reported for the *Botryococcus* genus, it should be highlighted that microalgae accumulation of lipids is a response to environmental and nutritional conditions, none of which have been imposed in the present work. Biodiesel production from microalgae lipids has been the focus of considerable attention in recent years [16,12]. Although high glycerol concentration has negatively affected total lipid content per cell for both species, biomass productivity was higher (25 and 50 mM). Thus, as a result, the final total amount of lipid increased in both cases (Table 2). Estimation shows that for treating 200 $m^3\ d^{-1}$ of wastewater it would be necessary 163 kg of glycerol in a year round treatment strategy (for both strains, Table 5). This represents an attractive solution for a cheap and unappreciated product.

The conversion of lipids into biodiesel is mostly carried out through alcoholic transesterification, and a well-established technology [46]. Therefore, several authors developed a strategy based on the assessment of fatty acids profiling for estimating final biodiesel quality [13,37,47]. Base on such an approach, it has been observed that *C. vulgaris* produced significant amounts of long chain isomeric formed FAs (C16 > C18 > C17, Table 4). The percentage of FA in the *C. vulgaris* profile was similar to the values reported elsewhere [6,4], with minor differences in the abundance of C18 isomers. Table 4 also shows the qualitative analyses of FAs (percentages of monounsaturated (MUFA), polyunsaturated (PUFA) and saturated fatty acids (SFA)). Glycerol at low concentrations favoured the synthesis of SFAs. At higher glycerol concentrations (25 and 50 mM) it was observed a considerable (205 and 122 kg y^{-1}, Table 5) shift in the FAs profile toward the synthesis of monounsaturated fatty acids (MUFA).

B. terribilis showed the highest ratio of lipid accumulation per biomass (25%), and, in average, productivity is comparable to that described elsewhere [48]. Other authors have registered similar results, with major differences in the amounts of FAs isomers of C16 to C18 [13,20,39]. At lower concentrations, glycerol did not significantly change the FAs-profiles. Mixotrophic condition at high glycerol concentration (50 mM) has increased FAs productivity (420 kg y^{-1}, Table 5) and the ratio of MUFA.

The biodiesel fuel quality depends on the results of the overall fatty acids composition present in the oil [13]. Several countries have already issued basic quality standards for biodiesel production and commercialization based on critical parameters such as: (i) Cetane Number (CN), (ii) Iodine Value (IV), (iii) Cold Filter Plugging Point (CFPP) and (iv) Oxidation Stability (OS). These parameters are a direct assessment of several important biodiesel properties such as ignition readiness, combustion performance, temperature of plugging fuel lines and biological stability during storage. A higher amount of long-chain-SFAs would lead to a biodiesel with elevated cetane number (CN). Biodiesel with higher CN values show a shorter ignition delay or a better combustion quality. Another parameter, which is also higher in oils rich in SFA, is the saponification values (SV). This refers to the average molecular weight of all FAs, which is measured by the quantity (mg) of potassium hydroxide required to saponify 1 g of fat. The saponification value varies with the chains-size, the longer the SFAs-chains are, the lower the SV. It is mostly associated with the presence of SFAs (C16, C17 and C18), but in this research saponification values increased for both strains at WW effluent amended with lower glycerol concentrations.

In this study the supplementation of glycerol into the microalgae growth medium has caused an increase in FAs saturation, which consequently resulted in positive alterations on CN, IV, OS. CN values (data not shown) variations were from 56 to 62 for *C. vulgaris* and from 58 to 67 for *B. terribilis*, respectively. All treatments are in compliance with the EN 14214 CN (minimum 51, Europe), ASTM D6751-10 (minimum of 47, USA) and RANP-2008 for CN (minimum of 45, Brazil). However the best (highest) CN values for *C vulgaris* was obtained in the condition of low glycerol concentrations (6.1 and 12.5 mM). In the case of *B. terribilis*, the best value was observed at 25 mM of glycerol supplementation. The calculated CFPP values were positive and showed to be similar to what has been previously reported for these strains [13]. Therefore, the increase in glycerol concentrations may result in a biodiesel with poorer low-temperature flow properties for both species, due to an increase in saturation of long-carbon-chains FAs. These FAs are the first to precipitate if liquid biodiesel is cooled down [13]. Yet, at 50 mM of glycerol supplementation the stearic FA concentration reduced significantly for both species (Table 4). Such results points toward an possible application of this treatment (50 mM glycerol) for the production of lipids with suitable quality to generate biodiesel for use at moderate temperature locations. It should be highlighted that there are several alternatives yet to be explored for improving the final FAs profiles and, therefore, change the po-

Table 4
Fatty acid (FA) profile of both strains at all treatments. Also shown the fatty acid qualitative distribution, total amount of FA in total lipids (TL) and FA percentage in the biomass (g per l00 g of biomass).

Fatty acids (%)		*Chlorella vulgaris*					*Botryococcus terribilis*				
		WW	WW + 6.1 mM	WW + 12.5 mM	WW + 25 mM	WW + 50 mM	WW	WW + 6.1 mM	WW + 12.5 mM	WW + 25 mM	WW + 50 mM
Butyric	C4:0	–	2.29	–	–	–	2.45	–	–	–	–
Caprylic	C8:0	1.41	–	–	–	–	1.92	–	–	–	–
Capric	C10:0		–	–	–	–	2,45	–	–	–	–
Undecanoic	Cll:0	0.87	–	–	–	–	–	–	–	–	–
Myristic	C14:0	2.06	–	–	–	–	2.60	–	–	–	1.70
Palmitic	C16:0	29.48	45.85	32.67	30.07	32.78	30.57	23.90	37.60	34.49	26.42
Palmitoleic	C16:l	1.96	–	–		17.06	5.73	4.07	5.89		2.46
Margaric/heptadecanoic	C17:0	7.04	24.84	25.34	2.73		8.16	9.95	15.46	16.62	11.44
Heptadecenoic	C17:l	5.65	–	–	4.31	–	3.04	–	–	–	–
Stearic	C18:0	12.24	29.31	22.29	14.98	16.15	12.66	14.71	6.96	20.70	13.51
Oleic	C18:l c	11.68	–	9.66	17.59	14.85	18.37	18.00	23.12	28.19	29.21
Elaidic/octadecenoic	C18:lt	1.39	–	–	4.67	4.21	–	–	–	–	4.42
Linoleic	C18:2n6	13.22	–	10.04	17.24	14.95	9.28	7.32	11.51	–	10.84
α-linolenic	C18:3n3	10.24	–	–	8.42	–	–	7.44	–	–	–
Eicosapentaenoic	C20:5 n3		–	–	–	–	2.04		–	–	–
Tetracosenoic	C24:ln9	2.76	–	–	–	–	3.17	14.60	–	–	–
Fatty acids concentration											
Saturated (% FA/TL)		53.10	100.0	80.30	47.77	48.93	59.36	48.56	59.48	71.81	53.07
Monounsaturated (% FA/TL)		23.44	0.00	9.66	26.57	36.13	26.49	36.68	29.01	28.19	36.09
Polyunsaturated (% FA/TL)		23.46	0.00	10.04	25.66	14.95	14.15	14.76	11.51	0.00	10.84
FA total (mg/TL)		2.52	0.31	0.64	1.99	1.07	1.56	0.70	1.06	1.64	1.55
FA total (% biomass DW)		5.05	0.77	1.29	3.98	2.15	3.20	1.41	2.10	3.29	3.10

Table 5
Estimates of productivities for both strains in a scenario of WWTP effluent production of 200 $m^3\ d^{-1}$ and with 240 working days per year. The values were estimated from the values empirically observed in the present research (values in the table) and the biofuels production options were estimated according with specialized literature (reference given below the table).

Estimates	*Chlorella vulgaris*					*Botryococcus terribilis*				
	WW	WW + 6.1 mM	WW + 12.5 mM	WW + 25 mM	WW + 50 mM	WW	WW + 6.1 mM	WW + 12.5 mM	WW + 25 mM	WW + 50 mM
Biomass productivity (ton y^{-l})	2.77	2.60	2.23	5.17	5.69	10.79	4.92	4.96	8.84	13.58
Glycerol required (kg y^{-1})	–	20.2	40.9	81.8	163.6	–	20.2	40.9	81.8	163.6
Lipid productivity (kg y^{-1})	755.5	566.4	541.4	692.6	894.2	2697.6	469.4	1290.7	1472.6	1683.4
Total FA (kg y^{-1})	139.9	20.1	28.8	205.7	122.2	345.3	69.3	104.3	291.0	420.9
Total carbohydrates (kg y^{-l})	108.0	135.4	131.6	351.5	415.0	841.7	349.0	307.8	433.4	1072.6
Biodiesel from TL (kg y^{-1})[a]	755.5	566.4	541.4	692.6	894.2	2697.6	469.4	1290.7	1472.6	1683.4
Biodiesel from FA (kg y^{-1})[b]	139.9	20.1	28.8	205.7	122.2	345.3	69.3	104.3	291.0	420.9
Residual $glycerol_{from\ TL}$ (kg y^{-1})[c]	75.5	56.6	54.1	69.2	89.4	269.7	46.9	129.0	147.2	168.3
Residual $glycerol_{from\ FA}$ (kg y^{-1})[d]	13.9	2.01	2.88	20.5	12.2	34.5	6.93	10.4	29.1	42.0
Bio-ethanol (l y^{-1}) [e]	51.0	64.0	62.2	166.1	196.1	397.6	164.9	145.4	204.8	506.7
Methane (m^3 l y^{-1}) [f]	664.7	625.0	535.4	1240.6	1364.5	2589.8	1179.9	1191.5	2122.8	3258.5
Fertilizer (N kg y^{-1})[g]	277.0	260.4	223.1	516.9	568.6	1079.1	491.6	496.5	884.5	1357.7
Fertilizer (P kg y^{-1}) [h]	27.7	26.0	22.3	51.7	56.9	107.9	49.2	49.6	88.4	135.8

[a] Biodiesel production of 1 kg per kg of oil [16]. Here estimated based on full lipids conversion to methyl esters.
[b] Same reference as before. Here estimated based only in the conversion of free fatty acids.
[c] Glycerol generated in the biodiesel production from total lipids extracted from biomass (conversion rate of 0.1 kg per kg of oil feedstock).
[d] Glycerol generated in the biodiesel production from Fatty acids (FA) extracted from biomass (conversion rate of 0.1 kg per kg of oil feedstock).
[e] Ethanol conversion rate of 0.6 l per kg of total carbohydrates.
[f] Methane production rate of 240 l per kg of biomass [49].
[g] Accounted as 10% of average biomass dry weight [50].
[h] Accounted as 1% of average biomass dry weight [50].

tential biodiesel quality generated by such strains. This preliminary biodiesel quality estimation is only helpful as a means to improve culturing conditions and guarantee commercial application. Overall, this research has shown that mixotrophically grown *C. vulgaris* and *B. terribilis* can generate fatty acids that will produce a biodiesel with good quality.

For both strains, the condition that produced at the same time the highest FA productivity and MUFA concentration was the treatment with 50 mM of glycerol. Such results when applied to the production system show a very promising outcome, in which quantity and quality of biodiesel can be achieved simultaneously. This research has shown that mixotrophically produced fatty acids can generate a biodiesel with good quality for both strains. The estimation shows a production of lipids (FA) of about 122 or 420 kg per year. Although such values may not sound significant at first glance, this is the potential production of a small-scale operating plant.

4. Conclusion

This research shows the positive effect of coupling a WWTP effluent with glycerol for supporting mixotrophic production of *C. vulgaris* and *B. terribillis*. The best performances, assessed as biomass and fatty acid productivities, were achieved using mixotrophic growth at glycerol concentration of 50 mM. Likewise, the total composition of fatty acids in each strain at glycerol supplementation of 50 mM showed to be the best profile for the generation of a good quality biodiesel. These findings identify this biological process as a very good alternative for combining wastewater treatment to algal biomass derived energy production. Another important advantage of such a process is a clear improvement on nutrient removal (N and P) to concentration levels below the threshold suggested by the EU directive. A year round productivity was estimated, showing that such a mixotrophic system can generate biomass yields of 5.6 and 13.5 tons y^{-1}, for *C. vulgaris* and *B. terribilis*, respectively. The volume of glycerol required for sustaining such a production is of about 163 kg y^{-1}. Several biorefinery options are available for microalgae biomass, but the biodiesel production is being considered as the most suitable for *C. vulgaris* and *B. terribilis*, respectively. Therefore, this research suggests that enhanced performance and profitability of a WWTP can be achieved coupling algal biomass production as energy feedstock with effluent post-treatment.

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