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Effect of nutrient availability on lipid productivity of *Botryococcus* sp. (Botryococcaceae, Chlorophyta), a newly isolated tropical microalgae strain from Puerto Rico

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Abstract.–Microalgae are promising sources of biofuels due to the high lipid content of some species. However, growing microalgae at large scales involves high production costs, mainly associated with nutrient inputs and harvesting processes. Therefore, to be cost competitive, species to be used as a source of fuels should be capable of accumulating lipids and biomass at lower fertilizer inputs. In the present study, we isolated and identified a native microalgal strain of *Botryococcus* sp. which was cultured under varying nitrogen, phosphorus and carbon dioxide regimes. The effects of nutrient availability on biomass, lipid production and fatty acid profiles were examined. We observed an increase in the relative lipid content from 25.5% under nitrogen non-limiting conditions to 41.8% under nitrogen deprivation. The lipid profile induced by nitrogen limitation was found to be dominated by saturated and monounsaturated lipid classes, meeting the European Standards for biodiesel and oil suitable for biofuel production. Thus, this *Botryococcus* sp. has the potential to be used at large scale cultures with the purpose of producing biofuels with lower fertilizer costs.

The high consumption of fossil fuels, the decline of oil discoveries and the contaminants emitted to the environment, have made the continued use of fossil fuels almost unsustainable. This has led to the search of renewable alternatives for energy production to improve energy security and environmental sustainability (Suganya et a. 2016). One alternative that has the potential to significantly diminish the harmful emissions and to decrease the greenhouse, climatic changes and global warming effects is the use of biofuels (Milano et al. 2016).

Several feedstocks for biofuel production have been proposed. Among these, microalgae are considered an attractive option since they store excess solar energy derived from photosynthesis as lipids, with triacylglycerols (TAGs) as the main storage form and these lipids can be easily converted to biodiesel (Suganya et a. 2016). Also, some species have fatty acid profiles that allow for the production of biodiesel with high oxidation stability and properties such as density, viscosity, acid value and heating value, similar to those of diesel fuel (Gouveia and Oliveira 2009).

Compared to traditional oil derived from other sources, such as palm oil and soybeans, microalgae have several advantages. Due to their simple cellular structure, they have faster growth rates than higher plants, they can be cultivated in various climates and in areas not used for food crops, minimizing the impact of algal biomass production on agriculture (Converti et al. 2009; Griffiths and Harrison 2009). In contrast to land crops, microalgae more easily access water, carbon dioxide and other nutrients and are less affected by variables such as sunlight and temperature (Widjaja et al. 2009). As an added benefit, their production is not seasonal and they have short harvesting cycles, allowing significant increased yields (Schenk et al. 2008), and in culture they are highly productive (Chen et al. 2011). Several species can accumulate high proportions of lipids and this has made microalgae-based biodiesel very attractive, (e.g.) there have been reported microalgal lipid productivities 15–300 times higher than that of conventional crops (Xin et al. 2010). High lipid yield is an ideal characteristic for a microalga to be considered as a feedstock for biofuel production in a cost competitive manner.

Lipid yield in microalgae is influenced by growth rates and environmental conditions (Xin et al. 2010). Thus, changes in culture conditions that affect growth, will also alter their lipid content. Studies have shown that most microalgal species adapt to stressful growing conditions by producing large amounts of TAGs (Converti et al. 2009; Gouveia and Oliveira 2009; Chen et al. 2011). Under these stressful conditions the cell division cycle stops since protein biosynthesis is limited (Schenk et al. 2008). However, if the cell density is not too high to avoid light penetration, photosynthesis can continue and CO_2 fixation can be switched to the accumulation of energy storage molecules, (e.g. TAGs) rather than protein synthesis, resulting in a higher lipid content in the microalgal biomass (Schenk et al. 2008; Xin et al. 2010).

Some of the stressors that have demonstrated to affect growth and lipid accumulation in microalgae are light intensity, temperature, CO_2 diffusion, salinity and concentration of nutrients (Xin et al. 2010). The response to these stressors vary depending on the species and the particular environmental conditions (Griffiths and Harrison 2009; Huang et al. 2010). The limitation of nitrogen directly affects algal growth by decreasing protein synthesis and inducing TAG accumulation (Xin et al. 2010).

Several studies have examined the effect of nitrogen concentration on lipid content in microalgae. Han et al. (2013) observed a 3.6-fold increase in lipid productivity when culturing *Chlorella pyrenoidosa* under nitrogen limita-

tion. Converti et al. (2009) found an increase of 7% and 10% on the lipid fractions for *N. oculata* and *C. vulgaris* respectively, when nitrogen concentration decreased by 75% with respect to the optimal values for growth. Griffiths and Harrison (2009) reported a lipid yield of 138% under N deficient conditions, compared with N sufficient conditions. Widjaja et al. (2009) found N depletion to promote the accumulation of lipids and trigger a shift from free fatty acids to TAGs in *Chlorella vulgaris*.

Phosphorus availability has also shown to affect lipid content in some species (Brennan and Owende 2010; Juneja et al. 2013lipids)and it involves operational efficiency, minimisation of environmental impact and socio-economic considerations; all of which are interdependent. It has become increasingly obvious that continued reliance on fossil fuel energy resources is unsustainable, owing to both depleting world reserves and the green house gas emissions associated with their use. Therefore, there are vigorous research initiatives aimed at developing alternative renewable and potentially carbon neutral solid, liquid and gaseous biofuels as alternative energy resources. However, alternate energy resources akin to first generation biofuels derived from terrestrial crops such as sugarcane, sugar beet, maize and rapeseed place an enormous strain on world food markets, contribute to water shortages and precipitate the destruction of the world's forests. Second generation biofuels derived from lignocellulosic agriculture and forest residues and from non-food crop feedstocks address some of the above problems; however there is concern over competing land use or required land use changes. Therefore, based on current knowledge and technology projections, third generation biofuels specifically derived from microalgae are considered to be a technically viable alternative energy resource that is devoid of the major drawbacks associated with first and second generation biofuels. Microalgae are photosynthetic microorganisms with simple growing requirements (light, sugars, CO2, N, P, and K. Xin et al. (2010) found phosphorus limiting conditions to induce lipid accumulation to about 53% of the algal biomass in *Scenedesmus* spp. Similarly, Khozin-Goldberg and Cohen (2006) found TAG levels increasing from 6.5% up to 39.3% of total lipids under phosphorus limitation in *Monodus subterraneus*.

The biomass and lipid production of several microalgal species have been also examined in respect to the availability of carbon dioxide, an essential nutrient since most of the microalgal species are photosynthetic (Brennan and Owende 2010)and it involves operational efficiency, minimisation of environmental impact and socio-economic considerations; all of which are interdependent. It has become increasingly obvious that continued reliance on fossil fuel energy resources is unsustainable, owing to both depleting world reserves and the green house gas emissions associated with their use. Therefore, there are vigorous research initiatives aimed at developing alternative renewable and potentially carbon neutral solid, liquid and gaseous biofuels as alternative energy resources. However, alternate energy resources akin to first generation biofuels derived from terrestrial crops such as sugarcane, sugar beet, maize and rapeseed place an enormous strain on world food markets, contribute to water shortages and precipitate the destruction of the world's forests. Second generation biofuels derived from lignocellulosic agriculture and forest residues and from non-food crop feedstocks address some of the above problems; however there is concern over competing land use or required land use changes. Therefore, based on current knowledge and technology projections, third generation biofuels specifically derived from microalgae are considered to be a technically viable alternative energy resource that is devoid of the major drawbacks associated with first and second generation biofuels. Microalgae are photosynthetic microorganisms with simple growing requirements (light, sugars, CO2, N, P, and K. The availability of CO_2 can influence microalgae growth and biomass production and consequently can also affect lipid accumulation.

In spite of the growing body of literature on microalgae lipid production, few studies have been conducted in the Caribbean region. Particularly in Puerto Rico, some inventories of microalgal species have been performed but no indigenous strains have been fully characterized in terms of lipid productivity. The geographical location of Puerto Rico allows large daily periods of light and high radiation favoring microalgae growth; thus, characterizing native strains is important, as it could allow for the isolation of candidates for efficient biofuel production already adapted to an ideal environment for large scale culturing. In the present study, we isolated a native microalgae strain from a brackish pond in Puerto Rico, and cultured it under different N, P, and CO_2 regimes. Changes in biomass, lipid content and lipid profiles triggered by some of these conditions were examined. The feasibility of scaling-up was examined by culturing *Botryococcus* sp. in a tubular photobioreactor in the presence of CO_2 and in a semi-continuous mode. Finally, a cost-benefit analysis of microalgae lipid production was conducted, to determine the cost of growing microalgae at commercial scales.

MATERIALS AND METHODS

Microalgae isolation and identification

One liter samples from a brackish pond in Dorado, Puerto Rico, were collected in sterile plastic bottles and kept at 8°C until processing. One hundred mL were filtered through a 47 mm nitrocellulose membrane filters (Pall Corp., pore size 0.45 μm). The filters were immersed in CHU 13 culture medium and incubated at room temperature and constant light to have a well-established culture. To isolate single species, serial dilutions of the mixed cultures were performed, and these were transferred to agar plates of the same culture medium. Plates were incubated under the same conditions until colonies were visible. Individual colonies were transferred to 200 μL of culture media and gradually scaled up until reaching a volume of 200 mL. Purity of the culture was constantly evaluated by microscopic observation.

The isolated species was identified by sequencing of the 18S small ribosomal subunit gene. DNA was extracted

following the protocol by Fawley and Fawley (2004), but with some modifications. Briefly, one mL of the culture was centrifuged at 5600 xg/1 min. The supernatant was discarded and 600 µL of extraction buffer (1 M NaCl, 70 mM Tris, 30 mM Na 2 EDTA, pH 8.6) were added to the pellet. The mixture was vortexed, and the tube was subjected to sonication (Branson Ultrasonic Bath Model 1200) for 10 min, vortexed every 2-3 min during the sonication period, and then transferred to a fresh tube containing 0.3g ± 0.01 g of glass beads (G-8772, Sigma Chemical Co., St. Louis) and agitated in an Analog Vortex Mixer (VWR) at the maximum speed for 3-5 min. The tube was centrifuged at 5600 xg/1 min and the supernatant was transferred to a new tube. Twenty-five µL of CTAB 10% and 200 µL chloroform were added. The organic and aqueous phases were separated by centrifugation at 2000 g/ 2 min and the aqueous phase transferred to a new tube. The DNA was purified using a Wizard DNA Clean-Up system (Promega), quantified using a Nanodrop ND-1000 spectrophotometer and amplified by Polymerase Chain Reaction (PCR). The following primers, specific for Chlorophyta species (green algae) and previously reported by Zhu et al. (2005), were used: EUK 528f–CCGCGGTAATTCCAGCTC and CHLO02r-CTTCGAGCCCCCAACTTTC.

PCR conditions were as follows: 2 min of denaturation at 95°C, 35 cycles of 45 s at 94°C, 45 s at 56°C (annealing temperature) and 1 min of extension at 72°C, and a final primer extension cycle of 7 min at 72°C. The amplicons were sequenced by Sanger sequencing and resulting sequences were compared to public databases (BLAST).

Growth conditions

Botryococcus sp. culture was inoculated in CHU 13 medium and maintained at 25°C under continuous cool-white fluorescent illumination at an incident intensity of 50 µmol photons m−2 s−1 (PAR) with orbital shaking at 60 rpm (Lab-line Orbit Shaker No. 3595). These conditions were maintained until reaching late exponential phase (i.e. 3 weeks). The duration of the exponential phase of growth was calculated in preliminary experiments in which population increases were determined daily by microscopic counts using a chamber.

Experimental design

For the experiment on the effect of nitrogen concentration, a late exponential growth phase culture of *Botryococcus sp.* was concentrated by centrifugation at 4000g for 5 min. The supernatant was discarded and the pellet washed with KNO_3-free CHU 13 medium.

Four levels of KNO_3 were established, each one with a set of five replicates, in a volume of 250 mL: (i) 4 mM (400 mg KNO_3/L), i.e. the concentration of nitrogen present in the original CHU 13 culture media, this non-N limited culture was used as a control; (ii) 2.0 mM (200 mg KNO_3/L); (iii) 0.9 mM (90 mg KNO_3/L); and (iv) KNO_3-free culture. Each replicate was inoculated with the concentrated inoculum.

For the experiments on the effect of phosphorus concentration, the same procedure was followed, but inoculating the late exponential growth phase culture in P-free CHU 13 medium at which four levels of K_2HPO_4 were added: (i) 0.45mM (80 mg K_2HPO_4/L), i.e. the concentration of phosphorus in the original CHU 13 culture media (control); (ii) 0.135 mM (24 mg K_2HPO_4/L); (iii) 0.045 mM (8 mg K_2HPO_4/L); and (iv) a K_2HPO_4-free culture.

These concentrations were selected based on previous studies showing lipid content increases when culturing microalgae at a N concentration 4.4 times lower than that in the full medium (Yamaberi et al. 1998) and a P concentration 10 times lower than that in the full medium (Khozin-Goldberg and Cohen 2006). To have the same initial inoculum, each replicate was adjusted to an optical density of 0.05 at 570 nm with a spectrophotometer (equivalent to 5 x 10^5 cells/mL). Replicates were incubated at room temperature, constant light and agitation for 3 weeks.

Biomass and growth rate determination

Biomass was examined every two days by dry weight determination and cell counts using a hemocytometer and the growth rate (K) was calculated as follows (Doan et al. (2011):

$$K=\ln N2-\ln N1\Delta t$$

Where N_1 and N_2 are the biomass measures at time 1 (t_1) and time 2 (t_2) respectively. The number of divisions per day and the generation or doubling time, were calculated in the following way:

$$Divisions\ per\ day=K\ln 2$$

$$Generation\ time=1/\text{Div. per day}$$

Lipid content determination and lipid composition analysis

A 10 mL aliquot was harvested every two days and centrifuged at 13751 xg for 10 min. The supernatant was discarded and the pellet dried at 60°C/10 min. Lipid extraction was conducted by adding 10 mL of hydranal (Chloroform: Methanol, 2:1), followed by sonication for 15 minutes (Branson Ultrasonic Bath Model 1200) to completely disrupt the cells. Microscopic observation was used to assess the disruption of intact cells (Mcmillan et al. 2013). After sonication, sterile nanopure water was added to separate the organic and aqueous phases. The organic phase was transferred to a previously weighted vial, evaporated and weighted again.

The resulting lipids were derivatized by the addition of N,O-bis (trimethylsilyl) trifluoroacetamide and trimethylchlorosilane (BTSFA-TMCS) as the silylating agent. After the derivatization procedure, the samples were diluted to 2 mL with dichloromethane. An aliquot of 1 µL was analyzed by GC/MS with helium as a carrier gas. The GC/MS was done as follows: sample injection split less, inlet temperature at 250° C, pulse pressure 25 psi, and flow 1mL/ min; Column DB-5 30 m x 0.320 mm and film 0.21 μm. The MS was in positive ion and total ion scan mode. The GC oven temperature program was as follows: held at initial temperature at 70° C for 1 min, then 5° C /min until 120° C, followed by an increase of 8° C/min until 260° C were it was held for 5

min. Compounds were identified using a NIST library; only compounds with a quality identification (R match) of ≥70% and a Signal to noise (S/N) ratio equal or higher to 15 were reported. Total fatty acid concentration was calculated as the sum of all individual fatty acids.

Statistical analysis

To evaluate the effect of N and P concentrations on biomass and lipid content, repeated measures ANOVA was performed using the SPSS statistical software.

Culture of Botryococcus *sp. at a medium scale in the presence of carbon dioxide*

Botryococcus sp. was cultivated in a tubular photobioreactor to evaluate the feasibility of scaling-up this species as well as its performance when cultured in the presence of CO_2 and in a semi-continuous mode. Bioreactors (67 L) were filled with CHU 13 culture media and inoculated with an exponential phase *Botryococcus* sp. Inoculum volume was added to provide 10% of the expected final volume. Light was supplied externally with cool-white fluorescent lamps at an incident intensity of 50 µmol photons m^{-2} s^{-1} (PAR). The lamps were installed 20cm from the bioreactor to provide a short light path and allow the dark zones to be maintained at a minimum. CO_2 was injected through a diffuser with a small-size bubble capacity. Large sterile air bubbles were injected separately to provide both aeration and mixing.

The cultivation started as a batch culture with a volume of 66 L. The semi-continuous mode was initiated at day 6, by removing a portion of the volume and replacing it with fresh culture media. Four cycles of culture withdrawal and fresh medium addition were carried out. Biomass (as cell counts/mL) and lipid content were determined daily as previously described.

Cost-benefit analysis of microalgae lipid production

To determine the cost of growing microalgae at commercial scale, biomass and lipid productivity observed in the nitrogen and phosphorus experiment as well as in the photobioreactor experiment, were related to the cost of N, P, and CO_2 sources, assuming a volume of 1000 L. Values of biomass and lipid content from the day of maximum productivity were used to calculate the number of harvesting cycles

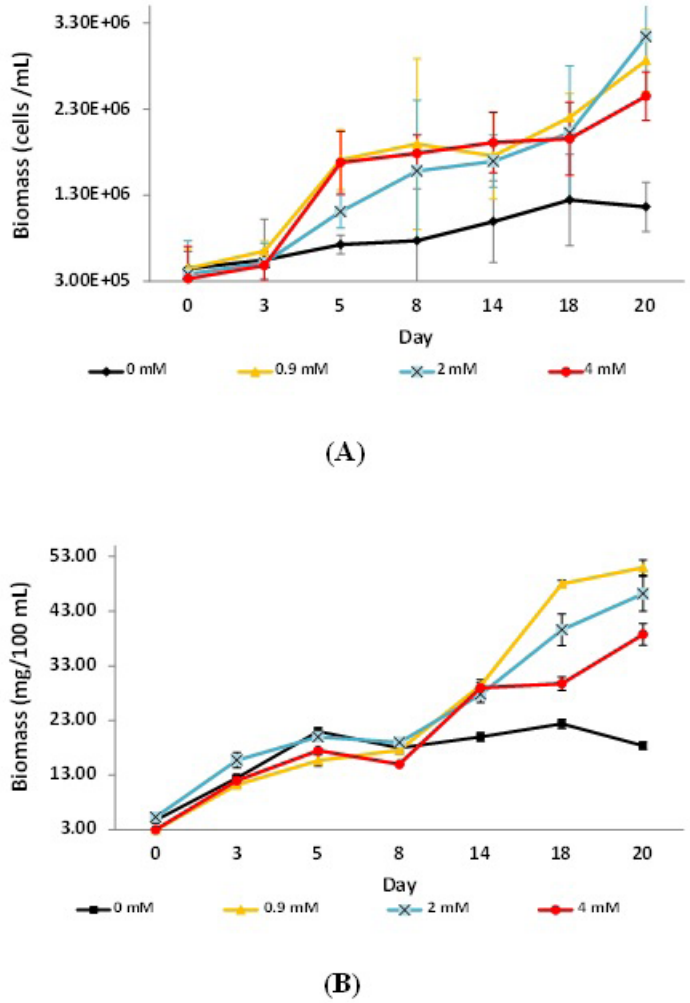

FIG. 1. Biomass changes in *Botryococcus* sp. under different nitrogen concentrations. A. Biomass changes as cells/mL; B. Biomass changes as mg/100mL.

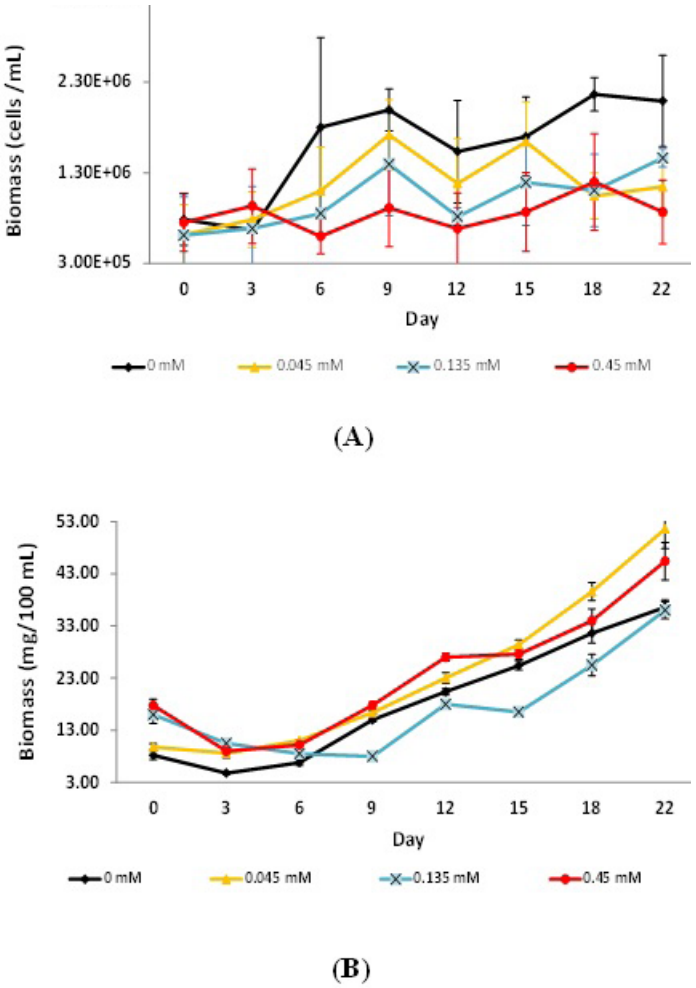

FIG. 2. Biomass changes in *Botryococcus* sp. under different phosphorus concentrations. A. Biomass changes as cells/mL; B. Biomass changes as mg/100mL.

per year (i.e. a harvesting cycle was defined as the number of days needed to reach the maximum productivity plus 2 days between one cycle and the next one).

Nitrogen and phosphorus were assumed to proceed from KNO_3 and K_2HPO_4, with a cost of \$0.73/kg and \$1.6/kg, respectively and the cost of CO_2 used for this analysis was \$0.22/kg (values taken from Beal et al. 2012b)algal biofuel research and development efforts have focused on increasing the competitiveness of algal biofuels by increasing the energy and financial return on investments, reducing water intensity and resource requirements, and increasing algal productivity. In this study, analyses are presented in each of these areas\u2014costs, resource needs, and productivity\u2014for two cases: (1. The total lipid production per cycle was calculated by multiplying lipid productivity (mg L^{-1} d^{-1}) by the number of days per cycle and by the total volume of the tank (L). Results were expressed in grams. This value was multiplied by the number of cycles per year to calculate the annual lipid production. Finally, the total cost of growing microalgae was calculated by multiplying the cost per cycle by the number of cycles per year.

Results and Discussion

Biomass and growth rates

Limited N concentrations reduced the overall growth rate of *Botryococcus* sp. When cultured at 2 mM a growth rate decrease of 25% was observed, whereas a nitrogen concentration of 0.9 mM led to a growth rate decrease of 43%. Nitrogen depletion supported a high growth rate until the third day, after which, the cell growth slowed, leading to an overall growth rate reduction of 45%. Accordingly, the generation time increased with decreasing N concentrations (Table 1). During the first three days of the incubation period, biomass (as cell counts/mL) was similar in the four N treatments, suggesting that at that point cultures were not limited by N. At day three, cell counts decreased in the N-absent culture, but remained similar in the N-reduced and N-sufficient cultures until day 18. From day 18 to day 20, cell counts in the two reduced N concentration cultures surpassed the cell number the N-sufficient culture (Fig. 1A).

According to Liebig's Law of the Minimum, the rate of production is limited by the least available resource, in this case nitrogen. The differences in biomass production at day 3 could be a consequence of the depletion of intracellular N storages in the N-absent culture. The fact that the two N-reduced concentrations supported growth during all the incubation period, suggesting that under the particular conditions of this study these N concentrations were not limiting. Results from biomass productivity and the statistical test run ($p > 0.05$) support this idea (Table 2).

The maximum biomass production observed in the nitrogen experiment was 0.55 g L^{-1}, equivalent to a biomass productivity of 27.8 mg $L^{-1}day^{-1}$. The minimum biomass production was 0.184 g L^{-1} or 9.2 g $L^{-1}day^{-1}$, observed in the N-absent culture (Table 2). These results are similar to previous findings. For instance, Yoo et al. (2010), found a biomass productivity of 26.55 mg $L^{-1}day^{-1}$ when culturing *Botryococcus braunii* in the presence of CO_2 and 55.36 mg N L^{-1} as the initial N concentration in the culture media. Also, Lee et al. (2010) cultured *B. braunii* in BG11 medium containing an initial N concentration of 247 mg L^{-1} and found a biomass productivity of 35.7 mg $L^{-1}day^{-1}$. In the present study the higher biomass productivity was reached in one of the N-reduced concentrations (i.e. 2 mM) in which the initial N concentration was 27.68 mg L^{-1}. This is in accordance with the general N requirement for green algae (5 to 59 mg L^{-1}; Becker 1994), although this values varies with species. To determine the appropriate nitrogen range for optimal growth of *Botryococcus* sp., as well as the concentrations of N that limits growth, it would be necessary to culture this strain under N concentrations below 5 mg L^{-1}.

The observed biomass production is low in comparison with other Chlorophyta species, such as *Scenedesmus* spp. and *Chlorella* spp. (Yoo et al. 2010; Rowley, 2010). However, higher growth rates were observed when culturing this strain in a photobioreactor with bubbling carbon dioxide. This suggests a higher biomass potential for *Botryococcus* sp. under such conditions.

When culturing *Botryococcus* sp. under the four phosphorus concentrations tested, a positive effect of P limitation on the overall growth rate was found. Increases of 7%, 40% and 83% were found for 0.135 mM, 0.045 mM and 0% concentrations, respectively, in comparison with the P-replete culture conditions (Table 3).

As observed in the N experiment, during the first 3 days of incubation none of the cultures were limited by P, since the number of cells was similar among the four P treatments. From day 3 on, biomass in the P-sufficient culture decreased,

Table 1. Growth parameters of *Botryococcus* sp. at different KNO_3 concentrations.

KNO_3 concentration (mM)	Overall growth rate	Growth rate changes (%)	Generation time (days)
4	0.1605 ± 0.065	100%	4.7427±1.595
2	0.1201 ±0.041	75%	6.3691±2.26
0.9	0.0921 ±0.013	57%	7.6345±0.973
0	0.0877 ±0.041	55%	9.675±4.931

Table 2. Biomass production and biomass productivity of *Botryococcus* sp. under the four Nitrogen regimes.

	0 mM	0.9 mM	2 mM	4 mM
Biomass production (g L-1)	0.184 ± 0.06	0.51 ± 0.13	0.557 ± 0.19	0.483 ± 0.07
Biomass productivity (mg L-1 d-1)	9.2 ± 3.29	25.5 ± 6.91	27.8 ± 9.8	24.1 ± 3.61

TABLE 3. Growth parameters of *Botryococcus* sp. at different K_2HPO_4 concentrations.

K_2HPO_4 concentration (mM)	Overall growth rate	Growth rate changes (%)	Generation time (days)
0.45	0.0881 ± 0.0361	100%	7.7218 ± 2.7823
0.135	0.0946 ± 0.05	107%	7.4203 ± 3.6878
0.045	0.1236 ± 0.041	140%	5.5464 ± 1.6637
0	0.161 ± 0.068	183%	4.9262 ± 1.9072

whereas in the P-reduced and P-free cultures an increase was observed. This increase was more marked in the P-free culture (Fig. 2A). Significant differences in the number of cells mL^{-1} ($p = 0.02$) were observed between one of the reduced P concentrations (i.e. 0.135 mM) and the P-free culture. Other studies have demonstrated that P does not negatively influence the growth of *Botryococcus* spp. For instance, as early as 1942, Chu cultured *Botryococcus* spp. under constant N concentrations and varying P concentrations ranging from 0.5 to 100 ppm and found no marked differences in growth, until P deficiency occurred (Chu 1942).

To explain why lower P concentrations supported higher biomass production in this study, it is necessary to consider the N: P ratio in each treatment. The N:P ratio determines the allocation of these elements within the cell and thus, depending on their external availability, these nutrients are used for cell growth or in the synthesis of storage compounds, such as lipids and starch (Fields et al. 2014). The proportion of N and P in phytoplanktonic species has been established to be 16:1 (Redfield ratio). However, there are deviations to the Redfield ratio, since the ideal N: P ratio is species-dependent, but as a rule, N is always needed in a higher proportion than P. For instance, N to P ratios ranging from 5 to 17 have been reported for different species of marine phytoplankton (Geider and Roche 2002) and ratios of 5 to 8 have been reported for freshwater species (Xin et al. 2010). When P is high relative to N, nitrogen utilization

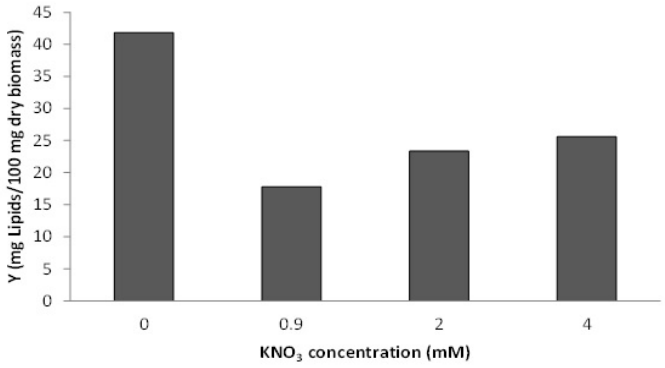

FIG. 3. Lipid yield (Y) of *Botryococcus* sp. at different KNO_3 concentrations (data shown are from the time point at which cellular fatty acid content was maxima).

TABLE 4. Biomass production and biomass productivity of *Botryococcus* sp. under the four Phosphorus regimes.

	0 mM	0.045 mM	0.135 mM	0.45 mM
Biomass production (g L^{-1})	0.366 ± 0.13	0.62 ± 0.34	0.36 ± 0.16	0.56 ± 0.3
Biomass productivity (mg L^{-1} d^{-1})	18.3 ± 6.97	24.8 ± 20.32	18 ± 8.04	28.12 ± 15.45

inside the cell is reduced before P requirement reach minimum. As a result, the medium became N limiting and the available P is not depleted (Rowley 2010). In this study, P never became depleted; thus, to assess the effect of P limitation, studies involving simultaneous N and P limitation should be conducted. Xin et al. (2010) cultured *Scenedesmus* spp. under N: P ratios ranging from 2:1 to 100:1 and found P not being influenced by the N: P ratio, since P decreased by 99% in all treatments. N: P ratios of 2 to 8 allowed an efficient removal of N, whereas higher ratios negatively influenced N utilization.

It has been shown that maximum phosphorus uptake rates are inhibited by high intracellular P content and thus the rate of uptake of P is dependent not only on the external P concentration, but also on the internal pool of polyphosphate (Spijkerman 2007). Thus, if a large reservoir of phosphorus exists, growth can be sustained under low external P availability (Bechemin et al. 1999). In this context, increases in P uptake rates have been observed in P-limited microalgal cells (Tomas 1979). Cembella et al. (1982) showed that this enhanced transport rate could be a consequence of a *de novo* synthesis of phosphatases involved in the active transport of P across the cell membrane or an activation of the already existing enzymes. Furthermore, the ability of storing amounts of nutrients higher than those required for immediate growth, a strategy known as "luxury uptake," has been observed in several species and can contribute to the increase in the intracellular P pool.

In a study of P uptake by *Chlamydomonas acidophila*, Spijkerman (2007) found an uptake rate 60-fold lower in P-saturated cultures, than in P-limited cultures and suggested that lower P concentrations could lead to higher growth rates in CO_2-aerated cultures. The minimum inorganic P concentration that supported growth was estimated to be 0.023 nmol Pi l^{-1} but higher concentrations (i.e. 17 to 43 nmol Pi l^{-1}) have been observed for other species (Spijkerman and Coesel 1996). The P concentrations used in this study are higher and as shown in Fig. 2, they were not really limiting, since growth was supported until the end of the incubation period.

The maximum biomass production observed in the P experiment was 0.62 g L^{-1}, equivalent to a biomass productivity of 24.8 mg $L^{-1}day^{-1}$. The minimum biomass production was 0.36 g L^{-1} or 18 g $L^{-1}day^{-1}$ (Table 4). The initial P concentration in the condition that yielded the maximum

biomass (i.e. 0.045 mM) was 1.42 mg L^{-1}.

Lipid content and lipid profiles

Our results showed that lipid content in *Botryococcus* sp. increased under N starvation conditions after 14 days of incubation. This is consistent with previous results, in which an increase in lipid content as a response to nitrogen limitation was observed for this genus. Zhila et al. (2005) found an increase of 21% in the total lipid content of *Botryococcus braunii* after 20 days of incubation under N-limiting conditions.

In this study, the absence of N in the culture media caused an increase in the relative lipid content from 25% to 42% (dry weight). In the two N-reduced cultures and in the N-sufficient one, the relative lipid content decreased. After 14 days of incubation, relative lipid accumulation became higher in N-absent culture than that in the N-sufficient culture. Figure 3 shows lipid yield (as mg lipids/100 mg dry biomass) in the four N concentrations at the day at which the highest cellular fatty acid content was observed. In the presence of stressors, metabolic changes induce the incorporation of carbon into the carbohydrate or lipid metabolism in microalgae instead of total biomass production (Fields et al. 2014). For instance, when the availability of N is low, the cell is unable to synthesize proteins required for cell division. Under these conditions, photosynthesis could be also reduced, but if light is available, it is not completely stopped. As a consequence, the cell must allocate the C fixed via photosynthesis into an alternative metabolic pathway such as lipid synthesis. Lipids can be easily catabolized to provide energy and thus are important energy storage molecules. Consequently, this metabolic shift allows microalgae to tolerate stressful conditions.

GC/MS analysis allowed us to characterize the lipid profile of *Botryococcus* sp. under the different nitrogen concentrations tested. This microalga showed an abundance of myristic (14:0), palmitic, (16:0), palmitoleic (16:1), palmitelaidic (16:1), stearic (18:0), Oleic (18:1) and linoleic (18:2) acids, which together comprised the 25% of lipids in the non N-limited culture and 42% in the N-absent culture (at the time point at which the highest lipid content was observed). Figure 4 shows the relative abundance of these fatty acids in the four N concentrations tested. Other lipid species were found, ranging from three carbon chain (Propionic acid) to 23 carbon chain (tricosylic acid). However, they were detected in very low concentrations (i.e. <0.5% w/w). This lipid profile is similar to that of vegetable oils traditionally used as a source of biodiesel, which are abundant in palmitic, stearic, linolenic, linoleic and oleic acids (Qu et al. 2012).

At the end of the incubation period, oleic acid was the most abundant lipid, comprising 61%, 28%, 61% and 75% of the overall lipid fraction in the N-sufficient, the two N-reduced and the N-free cultures, respectively. This was expected, since saturated and monoenoic acids are abundant during the stationary phase of microalgal growth (Zhila et al. 2005). Lipid composition did not vary among the different N concentrations tested. However, as shown in Figure 3 there was a variation in the total lipid content and this variation was triggered mainly by changes in the content of oleic acid. Oleic acid showed an increment in all N concentrations test-

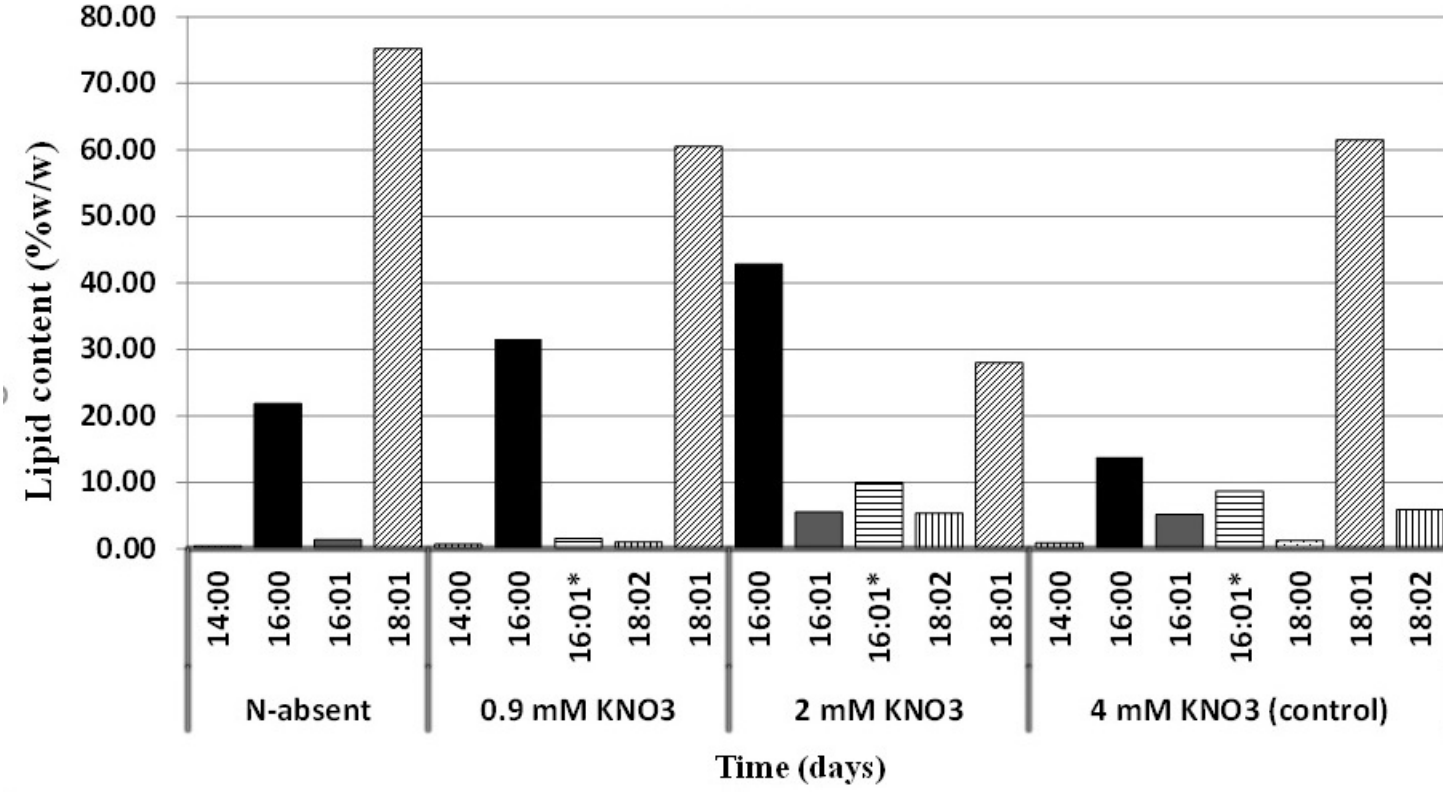

FIG. 4. Major lipids of *Botryococcus* sp. grown in the four N concentrations tested.

TABLE 5. Comparison of the average lipid composition at the first and last day of cultivation under N-sufficient, N-reduced, and N-absent conditions. Lipids found in a relative abundance <0.5% are not shown. Results are expressed as % of total fatty acids.

Major lipid compoents	4 mM KNO_3		2 mM KNO_3		0.9 mM KNO_3		0% KNO_3	
	Day 0	Day 20	Day 0	Day 20	Day 0	Day 20	Day 0	Day 20
Saturated								
C14:0	6.29	0.90	6.47	0	7.77	0.69	8.70	0.45
C16:0	34.20	13.78	32.29	42.8	43.56	31.57	44.86	21.86
C18:0			4.94	0	4.51	0	2.55	0.00
Unsaturated								
C16:1	13.51	13.86	11.17	15.59	17.93	1.59	14.41	1.40
C18:1	42.97	61.48	45.14	28.01	25.04	60.51	36.71	75.21
C18:2	0	5.91	0	5.35	1.19	1.07	0	0

ed, except in one of the N-reduced cultures (2 mM KNO_3). These changes were more pronounced in the N-free culture, than in the N sufficient one. For instance, an increase from 43% to 61% in the N-sufficient culture was observed, whereas in the N-absent culture it increased from 37% to 75% (Table 5). A high content of Oleic acid is a desired characteristic, since biodiesel rich in this fatty acid has a good ignition quality and low emissions of nitrogen oxides (NOx). Also, a high content of monounsaturated fatty acids increases the oxidative stability of biodiesel, which in turn, allows for longer storage periods (Qu et al. 2012).

Many microalgal species have lipid profiles rich in polyunsaturated fatty acids, which are more susceptible to oxidation. Ideally, biodiesel should not contain large quantities of unsaturated fatty acids, since this can affect biodiesel quality, especially during long storage periods. Also, lipids with a high degree of unsaturation solidify at low temperatures and thus biodiesel rich in these compounds cannot be used in engines (Schenk et al. 2008). In this study the content of linolenic and other polyunsaturated lipid species was less than 1% (w/w) under the four N concentrations tested, which is an ideal scenario, according to the European Standard for biodiesel (EN 14214).

Nitrogen limitation was also found to decrease the concentration of polyunsaturated lipids. After 20 days of incubation, the content of linoleic acid (18:2) in one of the N-reduced cultures (i.e. 0.9 mM KNO_3) was 5.5 times lower than that on the N-sufficient culture. The same pattern was observed with other polyunsaturated fatty acids such as cervonic acid (22:3), which although detected in low concentrations, was 6.7 times lower in the N-free culture, as compared to the N-sufficient one).

A shift towards the synthesis of saturated lipid classes instead of unsaturated fatty acids has also been observed in other green algae species under nitrogen starvation. For instance, Lohman et al. (2014), studied the fatty acid profile of *Chlamydomonas reinhardtii* and found the same proportion of saturated and unsaturated fatty acids (i.e. C16 lipid classes) under N replete conditions, but under N depletion, a fully saturated C16 fatty acid was preferentially synthesized. This shift from unsaturated to saturated lipids involves a hydrogenation step, which is an energy storage strategy for the algae. Also, long unsaturated fatty acids such as linolenic acid (18:3) are important components of cell membranes (Msanne et al. 2012) and as such, they are required for growth. As previously mentioned, under N-limitation microalgal cells reallocate C resources, diverting them from cell division towards the synthesis of N-poor energy storage compounds (i.e. TAGs). Finally, the unavailability of N cause a deficiency of aminoacids, which in turn, may prevent the synthesis of desaturases (responsible for the desaturation of fatty acids) and this could also favor the accumulation of saturated lipid classes.

Another important characteristic of biodiesel is the cetane number, i.e. an indicator of the ignition quality of the fuel, which is determined by the fatty acid distribution in the oil source. In general, the ignition delay period is shorter when biodiesel contains lipids with a high cetane number. In this sense, it has been suggested that biodiesel should ideally contain palmitoleic (16:1), oleic (18:1) and myristic (14:0) acids (Schenk et al. 2008). These lipids, together with stearic and linoleic acids were the most abundant fatty acids found in our experiments. Stearic and linoleic acids were not as abundant, suggesting that this oil composition could make *Botryococcus* sp. a suitable source of biodiesel. Comparable results were obtained when culturing *Botryococcus* sp. under different phosphorus concentrations (data not shown).

Culture of Botryococcus *sp. at a medium scale in the presence of carbon dioxide*

Both the bioreactor and the control experiments (*Botryococcus sp.* cultured at bench scale without CO_2) were inoculated with the same initial cell concentration (4.5 - 5 x 10^5 cells/mL). During the first 3 days, biomass was similar in both treatments. From day 3, biomass increased in the bioreactor as compared to the control (Figs. 5 and 6). As a result, large growth and lipid accumulation rates were recorded for *Botryococcus* sp. grown in the photobioreactor (Table 6).

Beginning at day six, a fixed volume was removed from the bioreactor at regular time intervals (i.e. every 6

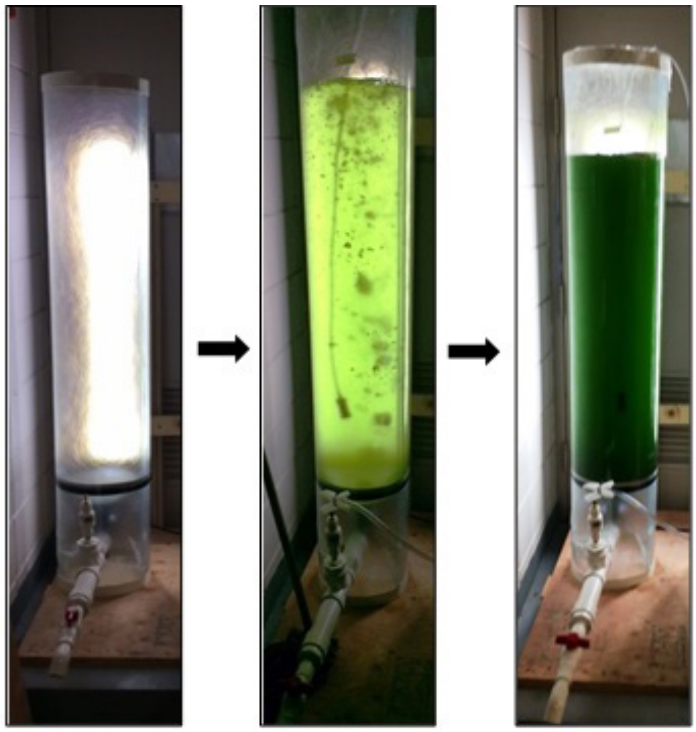

FIG. 5. Biomass increase in the photobioreactor from day 0 to day 5.

days) to harvest cells and an equal volume of fresh medium was immediately supplied to the culture. This medium addition decreased the number of cells, but enhanced nutrient concentration, thereby supporting a continuous growth over the incubation period (Fig. 7). The cell concentrations obtained at the time of harvesting (end of each cycle) were 8.9 x 10^6 cells/mL, 8.3 x 10^6 cells/mL and 6.5 x 10^6 cells/mL, respectively.

Given the colony-forming characteristics of *Botryococcus* sp. (Fig. 8), this microalga has a high sinking rate. Therefore, large air bubbles were injected to provide aeration and mixing. Mixing with large bubbles increase the light penetration depth and allow a homogeneous distribution of light inside the cultures, and thus, a higher exposition of each cell to light (Eriksen, 2008). This is important, since light limitation negatively affect biomass production. At the same time, the use of large bubbles ensures nutrient supply and elimination of products, such as oxygen, which in turn, may increase biomass productivity (Scarsella et al. 2012; Sobczuk et al. 2006).

The shear force introduced by mechanical mixing could negatively affect biomass production and this is species dependent (Scarsella et al. 2012), with large cells being more prone to turbulence-induced damage (Mazzuca and Chisti 2010). *Botryococcus* sp. cells are small in comparison with other Chlorophyta species such as *Chlorella* sp., and thus, are probably more tolerant to the shear stress induced by mixing in photobioreactors.

Although morphological traits have an influence in shear tolerance, the biochemical composition of the cell

TABLE 6. Growth and lipid accumulation rates of *Botryococcus* sp. cultured in a photobioreactor and in the presence of CO_2.

	***Botryococcus* sp. in culture media (control)**	***Botryococcus* sp. in bioreactor + CO_2**
Growth rate	0.1566	0.4929
Generation time (days)	4.43	1.41
Lipid accumulation rate	0.1905	0.3961

wall also appear to play an important role (Sobczuk et al. 2006). Species belonging to the genus *Botryococcus* have a relatively thick and rigid cell wall, compared to other green algae species. It is composed by an internal fibrillar layer made of polysaccharide and an external trilaminar sheath (Banerjee et al. 2002; Sakthivel et al. 2011), a structure that maintains the integrity of the cell and provide resistance to turbulent flows. *Botryococcus* sp. meets some characteristics considered ideal when culturing algae at large scale. Its morphology facilitates the harvesting process in column photobioreactors and its small cell size makes it resistant to the shear force induced by large bubble agitation.

Cost-benefit analysis of microalgae lipid production

As previously mentioned, the production of algal oil with energy purposes involves high costs, mainly associated with fertilizer inputs and harvesting processes. Taking into account the biomass and lipid production that resulted when culturing *Botryococcus* sp. at different nitrogen, phosphorus and carbon dioxide concentrations, projections of the cost of nutrient inputs needed at a commercial scale in open ponds were calculated (Table 7). The preparation of inoculum for the cultivation of microalgae at large scale (e.g. in open ponds) is usually accomplished in various stages, with the larger growth volumes being inoculated from the smaller growth volumes (Beal et al. 2012) algae were grown at several cultivation scales and processed using centrifugation for harvesting, electromechanical cell lysing, and a microporous hollow fiber membrane contactor for lipid separation. The separated algal lipids represent a biocrude product that could be refined into fuel and the post-extraction biomass could be converted to methane. To determine the EROI, a second-order analysis was conducted, which includes direct and indirect energy flows, but does not include energy expenses associated with capital investments. The EROI for the production process evaluated here was significantly less than 1, however, the majority of the energy consumption resulted from non-optimized growth conditions. While the experimental results do not represent an expected typical case EROI for algal fuels, the approach and end-to-end experimental determination of the different inputs and outputs provides a useful outline of the important parameters to consider in such an analysis. The Experimental Case results are the first known experimental energy balance for an integrated algal biocrude production facility, and as such, are

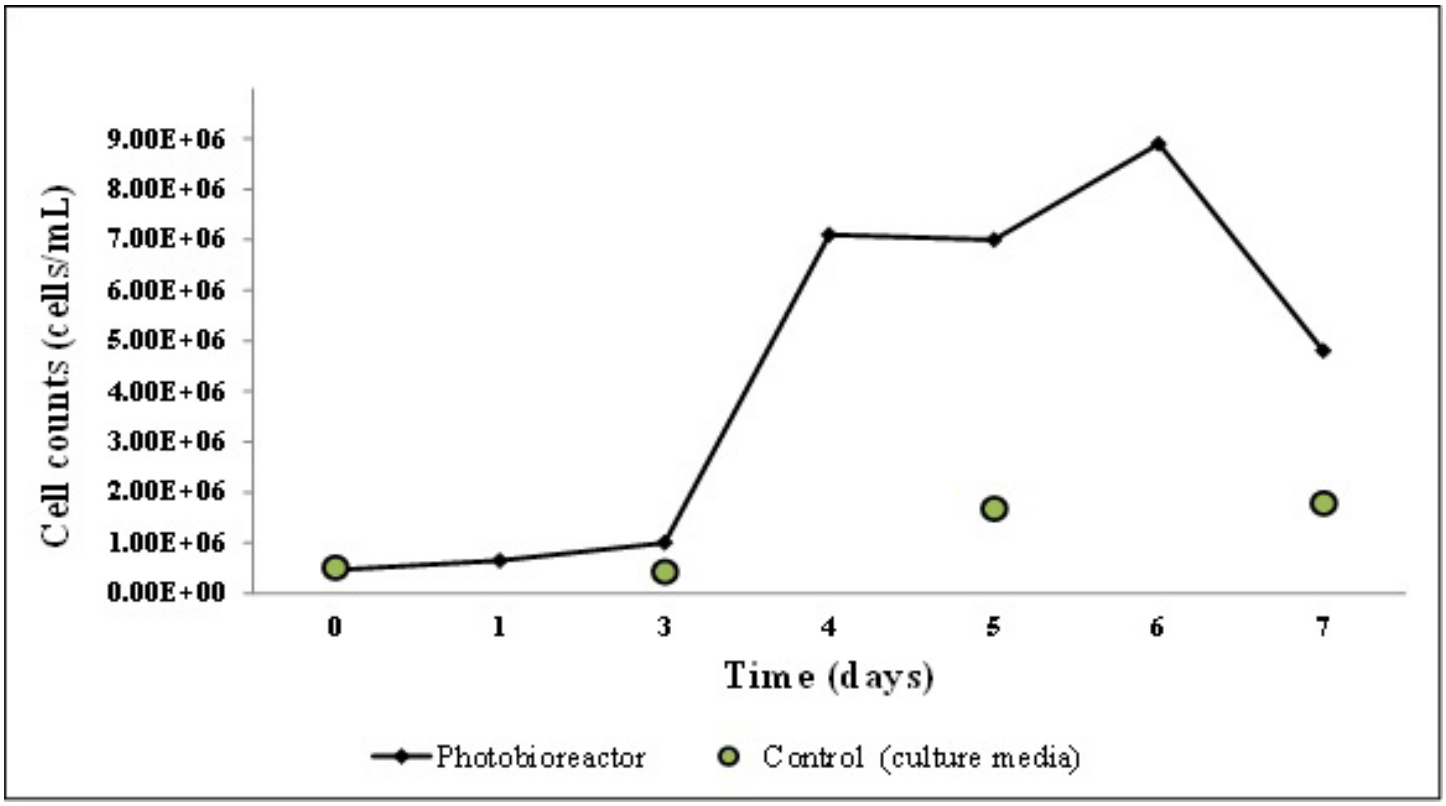

FIG. 6. Biomass changes during the first week of culturing *Botryococcus* sp. in a 67 L photobioreactor in the presence of CO_2 (line) compared with biomass changes under control conditions (green dots).

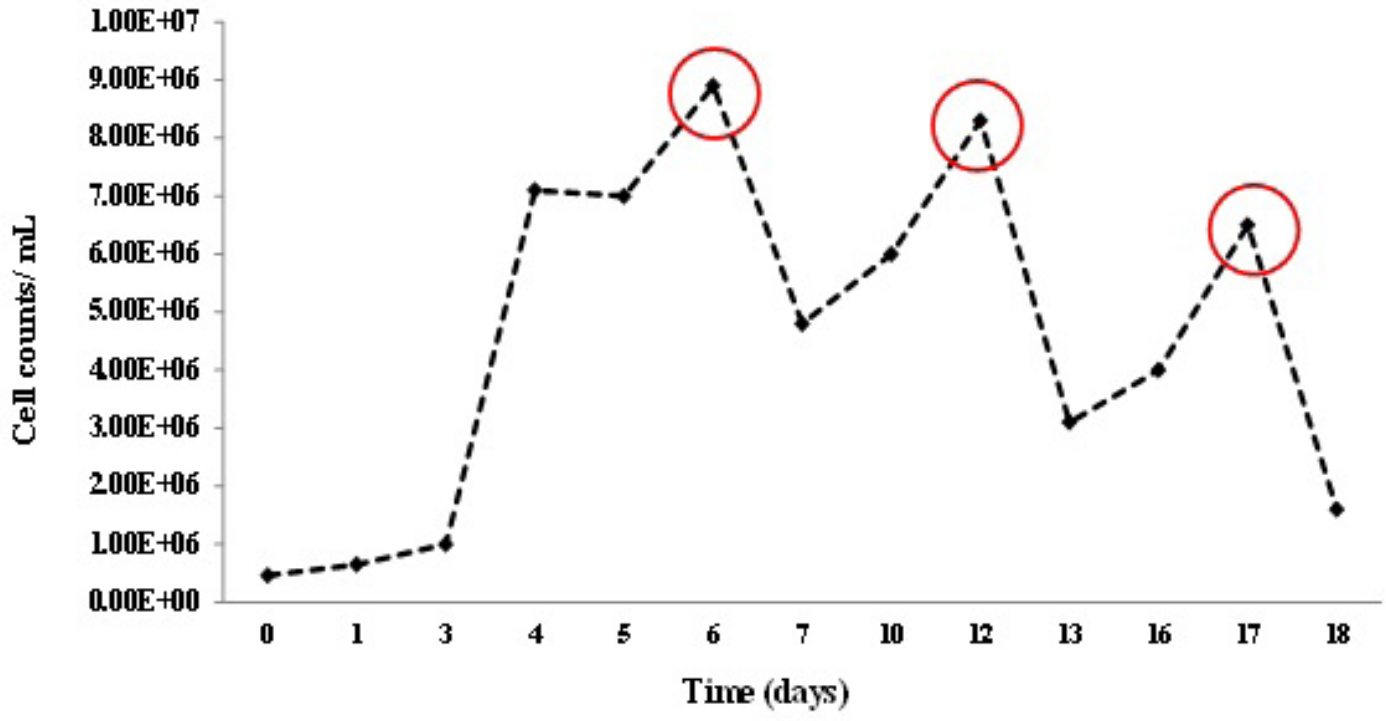

FIG. 7. Biomass changes of *Botryococcus* sp. cultured in a 67 L photobioreactor in the presence of CO_2 in a semi continuous system (red circles represent points in time at which cells were harvested and a same volume of fresh culture media was supplied).

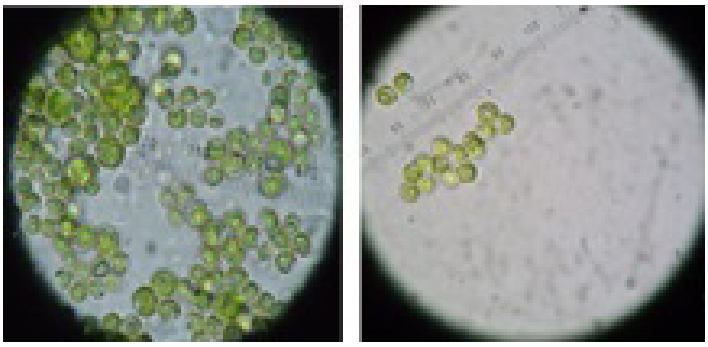

FIG. 8. *Botryococcus* sp. morphology.

expected to be helpful for setting research and development priorities. In addition to the Experimental Case (based on direct measurements. At first, microalgae are cultured at bench scales in volumes of 250 mL to 4 L, followed by intermediate scale cultures (i.e. > 50 L). A third stage involves the use of greenhouse tanks (volumes of about 1000 L) and finally, large-scale ponds are inoculated. Cost calculations were done based in greenhouse tanks of 1000 L.

Higher biomass and lipid productivity were reached when growing *Botryococcus* sp. with a constant influx of CO_2 and the full culture medium. Under these conditions, the cost associated with nutrient inputs is four times higher than the cost when CO_2 was not injected; however, it is important to take into account that these biomass and lipid productivity values were reached in a harvesting cycle as short as 4 days (compared with 14 to 20 days for the CO_2-free conditions). Among the three conditions in which no CO_2 was used, the full culture medium (N sufficient conditions in Table 7) showed higher lipid productivity (11.42 mg L^{-1} d^{-1}). To increase this lipid productivity to 32.5 mg L^{-1} d^{-1} (observed when CO_2 was used, last row in Table 7), three tanks of 1000 L would be needed. This represents an increase in the cost of nutrients from $9.6 to $27, as well as longer harvesting times.

Thus, a large-scale microalgae cultivation project should have CO_2 influx to reach high productivity in short periods. This analysis is based in one tank of 1000 L, but assuming a shallow pond of an area of 2000 m^2 and a depth of 30 cm (i.e. 600 m^3) 60 tanks of 1000 L would be needed to inoculate the pond, since inoculum must account for at least 10% of the volume of the cultivation pond (Murphy and Allen 2011). As a result, the total cost per year would be $2,263 for one pond. Assuming a farm of 1 ha (10,000 m^2) (5 ponds) $11,315 would be required. Therefore, to be competitive with conventional fuels, a microalgae-based biofuels project should ideally use discounted inputs. For instance, the use of treated wastewaters could reduce the cost of nutrients, while reducing the overall impact in wastewater disposal. Also, the utilization of flue-gas (exhaust gases from industrial and power generation plants) has been reported as a strategy to reduce the cost of CO_2 (Matsumoto et al. 1995).

By obtaining microalgal by-products, such as biofertilizers, bioplastics and biogas, among others, it would also be possible to achieve a higher earning potential, thus an increase in the processing costs may be justified. Also, the symbiotic production of aquaculture products (e.g. fish/shrimp) has been proposed. This analysis was conducted at the inoculant level (when conditions such as light source and maintenance of monocultures can be still be controlled). Other costs, such as those related with water use, energy, harvesting, etc., were not considered. Field studies should be conducted in the future considering these costs and larger scales.

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TABLE 7. Cost-benefit analysis of microalgal production.

	Nutrient concentration	Biomass productivity (mg L^{-1} d^{-1})	Lipid productivity (mg L^{-1} d^{-1})	Day of maximum productivity	Tank volume (L)	Harvesting cycles per year	N cost / cycle ($)	P cost / cycle ($)	CO2 cost / cycle ($)	Lipid production / cycle (g)	Total cost / cyle ($)	Annual lipid production (kg)	Total cost / year ($)
N sufficient conditions													
NO_3 concentration (mg L^{-1})	400												
PO_4 concentration (mg L^{-1})	80	20.71	11.42	14	1000	23	0.292	0.128	0	159.9	0.42	3.6	9.6
CO_2 concentration (lb day^{-1})	0												
N limitation conditions													
NO_3 concentration (mg L^{-1})	200												
PO_4 concentration (mg L^{-1})	80	23.1	10.00	20	1000	17	0.146	0.128	0	200.0	0.274	3.3	4.5
CO_2 concentration (lb day^{-1})	0												
P limitation conditions													
NO_3 concentration (mg L^{-1})	400												
PO_4 concentration (mg L^{-1})	8	22	7.78	18	1000	18	0.292	0.0128	0	140.0	0.3048	2.6	5.6
CO_2 concentration (lb day^{-1})	0												
Bioreactor exp													
NO_3 concentration (mg L^{-1})	400												
PO_4 concentration (mg L^{-1})	80	57.5	32.5	4	1000	61	0.146	0.128	0	200.0	0.274	3.3	4.5
CO_2 concentration (lb day^{-1})	0.5												

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