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A MULTISTAGE GRADUAL NITROGEN-REDUCTION STRATEGY FOR INCREASED LIPID PRODUCTIVITY AND NITROGEN REMOVAL IN WASTEWATER USING *Chlorella vulgaris* AND *Scenedesmus obliquus*

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Abstract - *Chlorella vulgaris* and *Scenedesmus obliquus* were grown in artificial-wastewater using a new nitrogen-limitation strategy aimed at increasing lipid productivity. This strategy consisted in a multi-stage process with sequential reduction of $N\text{-}NH_4$ concentration (from 90 to 60, 40, and 20 $mg.L^{-1}$) to promote a balance between cell growth and lipid accumulation. Lipid productivity was compared against a reference process consisting of nitrogen reduction in two stages, where the nitrogen concentration was suddenly reduced from 90 $mg.L^{-1}$ to three different concentrations (10, 20, and 30 $mg.L^{-1}$). In the multi-stage mode, only *C. vulgaris* exhibited a net lipid-productivity increase. Lipid content of *S. obliquus* did not present a significant increase, thus decreasing lipid productivity. The highest lipid productivities were observed in the two-stage mode for both *S. obliquus* and *C. vulgaris* (194.9 and 133.5 $mg.L^{-1}.d^{-1}$, respectively), and these values are among the highest reported in the literature to date.
Keywords: Nitrogen limitation; Lipid productivity; Multistage; Microalgae; Biodiesel.

INTRODUCTION

Biodiesel is one of the liquid biofuels that has received most interest due to its many advantages over fossil diesel. Currently, it is commercially produced from oleaginous plants, such as soy, African palm, and rapeseed. However, two major problems for the industrial production of biodiesel are feedstock availability, and land competition (direct or indirect) with food crops. As a response to these challenges, microalgae have been explored as a promising oil supply: they have higher photosynthetic efficiencies than terrestrial crops, exhibit higher biomass production rates per hectare, and can be used as a tertiary wastewater treatment. Many studies have shown that some microalgae species can store large amounts of triacylglycerides (TAGs) consisting of a mixture of saturated and unsaturated fatty-acid chains (C_{12} to C_{22}), which makes them appropriate for biodiesel production (Borowitzka, 1991).

Some microalgae species have high lipid content (20-50% of dry cell weight) and it is possible to increase it by controlling various biotic and abiotic factors of the culture, such as light intensity and in-

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termittence, temperature, salinity, CO_2 addition, agitation regime, and harvesting methods (Hu *et al.,* 2008; Chiu *et al.,* 2009; Widjaja *et al.* 2009). One of the most studied factors for increasing the lipid content is nutrient limitation; in particular, nitrogen and phosphorous limitation have been reported to increase lipid content and to shift the lipid composition towards TAGs (Widjaja *et al.,* 2009), although little is known about the underlying mechanisms (Li *et al.,* 2012). The microalgae *Chlorella* and *Scenedesmus* are often used in biodiesel-production studies, and they present the ability to grow in wastewater. Illman *et al.* (2000) reported that *Chlorella vulgaris* accumulated 14-30% w/w of lipids under N-sufficient conditions. The lipid content of *C. vulgaris* increased from 20% to 43% of dry cell weight under nitrogen starvation conditions (Mujtaba *et al.,* 2012). Scott *et al.* (2010) reported lipid contents of up to 70% for the same species, when cultivated under nitrogen deficiency. Furthermore, response to nutrient limitation has been shown to be species-specific (Griffiths *et al.,* 2011).

When grown under nutrient limitation, lipid content is expected to increase at the expense of cellular growth. This occurs because, under limited N concentration, protein synthesis required for cell growth is inhibited, leaving an excess of carbon from photosynthesis that is redirected to the metabolic paths of lipid storage and starch production (Li *et al.,* 2012; Scott *et al.,* 2010). Lipid productivity P_L (grams of lipids per liter of culture per day) is the most important variable to maximize the biodiesel production from microalgae cultures (Khozin and Cohen, 2006; Rodolfi *et al.,* 2009), since it represents the coupled effect of increased lipid content w (grams of lipids per grams of dry biomass) and reduced biomass concentration X (grams of dry biomass per liter of culture) in N-limited cultures.

Because of their high lipid productivity and adequate fatty-acid profile, *C. vulgaris* and *S. obliquus* have been identified as two species with great potential for biodiesel production (Griffiths *et al.,* 2011). Several works have reported lipid productivities of these two species as high as 180 $mg.L^{-1}.d^{-1}$ and 140 $mg.L^{-1}.d^{-1}$ for *C. vulgaris* and *S. obliquus*, respectively (Table 1). These productivity values were achieved under nitrogen-limited culture conditions (Widjaja *et al*., 2009; Mujtaba *et al*., 2012; Griffiths *et al.,* 2011; Gouveia and Oliveira, 2009; Feng *et al.,* 2011; Pranveenkumar *et al.,* 2012; Lv *et al.*, 2010; Ho *et al.,* 2010; Converti *et al.,* 2009; Ho *et al.,* 2012).

Using a two-stage cultivation mode, Rodolfi *et al.* (2009) grew *Nannochloropsis* sp. F&M-M24 in nitrogen-rich medium, and then diluted with fresh, low-nitrogen medium, increasing lipid productivity to 200 $mg.L^{-1}.d^{-1}$. On the other hand, Stephenson *et al.* (2012) reported that the most effective strategy to increase the productivity of TAGs in *C. vulgaris* was by continuing the cultivation until nitrogen depletion (in a single stage), rather than transferring the culture to fresh medium without nitrogen. Also, Mujtaba *et al.* (2012) reported a constant lipid content (14-16% w/w) and lipid productivity in *C. vulgaris* when growing the microalgae for 5 and 7 days using two cultivation modes: a single stage, and continuous addition of nitrogen in batch reactors. These results show that reducing the N concentration in the medium will result in an increase of lipid content in the microalgae, but the net lipid productivity may be the same, or even lower than in single batch culture, as a result of the reduction in the growth rate.

Furthermore, Feng *et al.* (2011) reported high lipid productivities in a semi-continuous culture of *C. vulgaris* in aerated-column reactors.

Table 1: Reported lipid productivities for *C. vulgaris* and *S. obliquus* in photoautotrophic cultures.

Species	P_L ($mg\ L^{-1}\ d^{-1}$)	Culture conditions and reference
C. vulgaris	180.0	Nitrogen limitation (Gouveia and Oliveira, 2009)
	147.0	Semi-continuous cultivation in bubble column photobioreactors (Feng *et al*., 2011)
	77.1	Nitrogen limitation (Mujtaba *et al*., 2012)
	67.0	Nitrogen limitation (Griffiths *et al*., 2011)
	54.0	Nitrogen starvation of *Chlorella* sp. BUM11008 (Praveenkumar *et al*., 2012).
	40.0	Optimized productivity limiting nitrogen source, adding CO_2 and varying the light intensity (Lv *et al*., 2010)
	16.9	Nitrogen starvation (Widjaja *et al.*, 2009)
	20.4	Reduced nitrate culture (Converti *et al*., 2009)
S. obliquus	140.4	Five-day nitrogen starvation in *S. obliquus* CNW-N (Ho *et al*., 2012)
	106.0	Nitrogen limitation (Griffiths *et al*., 2011)
	90.0	Nitrogen limitation (Gouveia and Oliveira, 2009)
	78.73	Nine-day nitrogen starvation in *S. obliquus* CNW-N (Ho *et al*., 2010)

They showed that 50% v/v dilutions with fresh medium every 24 h for three days, resulted in a lipid productivity of 147 $mg.L^{-1}.d^{-1}$, compared to 79 $mg.L^{-1}.d^{-1}$ obtained with daily replacement of 75% v/v with fresh medium. The lower lipid productivity in the second case was related to the reduced growth rates, since lipid content remained almost constant in both processes (38–42% w/w, respectively).

Based on all this evidence, a cultivation strategy consisting of sequential stages of gradual reduction of N concentration in the medium is analyzed in this study. In the design of microalgal-oil production processes it is apparent that lipid productivity is more important than lipid content only. Therefore, a strategy to increase the lipid productivity in autotrophic cultures may require balancing lipid content and growth rate through a multistage process, involving the gradual reduction of N concentration in the culture medium. Thus, two autotrophic cultivation modes of *C. vulgaris* and *S. obliquus* were compared: a multistage process (or gradual nitrogen reduction), and the most studied two-stage process (or sudden nitrogen reduction).

MATERIALS AND METHODS

Strain Selection and Culture Media

Strains of *Chlorella vulgaris* and *Scenedesmus obliquus* were obtained from the culture collection of the Centro de Investigación Científica y de Educación Superior de Ensenada (CICESE), Mexico. These species were selected due to their proven capacity to grow in wastewater, and having high N and P removal efficiencies (Ruiz-Marin *et al.* 2010). For the acclimation, both species were grown in culture media with a composition similar to that in the effluent of the primary treatment of an urban wastewater-treatment plant (Ruiz-Marin *et al.* 2010), as follows: 7 mg NaCl, 4 mg $CaCl_2$, 2 mg $MgSO_4 \cdot 7H_2O$, 15 mg KH_2PO_4, 115.6 mg NH_4Cl, all dissolved in 1 L of distilled water. Trace metals and vitamins were added according to the guidelines for medium f/2 (Guillar and Ryther 1962). During acclimation (1 month), both microalgae were transferred to fresh culture media every seven days at 28±1 °C and light intensity of 100 $\mu E.m^{-2}.s^{-1}$.

Two-Stage (TS) Nitrogen Reduction Cultures

The cultures were operated in 3 L cylindrical bioreactors made of transparent polyethylene terephthalate (PETE) containing 2 L of operational volume of culture medium. The reactors were pre-washed with a chlorine solution to prevent bacterial contamination. The treatments were run with an initial cell density of 2×10^6 $cells.mL^{-1}$ and aerated at 0.4 $L.L^{-1}.min^{-1}$, under a continuous illumination regime at 120 $\mu E.m^{-2}.s^{-1}$ (cool-white fluorescent lamps) and at a constant temperature of 28 ± 1 °C.

In most studies using a two-stage method for nutrient limitation, the first stage is a nutrient-rich culture with the purpose of achieving a high biomass concentration, while depleting the nitrogen in the medium; for this reason, the biomass is harvested at the end of the logarithmic phase of growth (centrifugation and precipitation being the most commonly used methods). The harvested biomass is then resuspended in fresh medium containing a low concentration or absence of nitrogen (Widjaja *et al*., 2009; Griffiths *et al.,* 2011; Converti *et al.,* 2009; Li *et al.,* 2008). For practical purposes, especially in larger capacity reactors, it is more convenient to replace the depleted medium from the first stage with fresh medium containing a lower initial nitrogen-concentration (Converti *et al.,* 2009; Li *et al.,* 2008; Zhila *et al.,* 2005; Tan and Lin, 2011). In this study, the latter approach was taken: the microalgae were first grown in a nitrogen-rich medium (90 $mg.L^{-1}$ in the first stage), using an operational volume of 1 L; at the end of the logarithmic phase of growth, 1 L of fresh medium was added (in order to achieve a 50% v/v dilution of the biomass). The residual nitrogen from the first stage was taken into account for adjusting the nitrogen concentration in the fresh medium in order to achieve initial concentrations in the second stage of 30, 20, or 10 $mg.L^{-1}$ of $N\text{-}NH_4$. At the end of each stage, measurements were made to determine nitrogen and biomass concentration and lipid content, as described in the following sections.

Multi-stage (MS) Nitrogen Reduction Cultures

The experimental system was the same as in the TS cultures. Initially, stock suspensions of *C. vulgaris* and *S. obliquus* of 2×10^6 $cells.mL^{-1}$ were grown under nitrogen-rich conditions (90 $mg.L^{-1}$ $N\text{-}NH_4$). After six days, the second stage was initiated by replacing 50% v/v of the depleted medium, and the N-concentration in the fresh medium was adjusted to achieve 60 $mg.L^{-1}$ of $N\text{-}NH_4$ in the resulting medium. This procedure was repeated twice more at the end of each exponential phase of growth, starting a third and a fourth stage with initial $N\text{-}NH_4$ concentrations of 40 and 20 $mg.L^{-1}$, respectively.

Determination of N-NH_4 Content, Cell Density, and Cell Dry-Weight

Every 12 h, 50 mL of water sample was collected for analysis. The concentration of N–NH_4 was determined according to standard methods (APHA, 1995). The number of algal cells was determined using a Neubauer chamber Hemacytometer 0.1 mm in depth. For the determination of ash-free dry weight, 20 mL of culture was filtered through a GF-C glass fiber filter previously rinsed with distilled water, and incinerated at 470 °C for 4 h. The samples were dried at 120 °C until constant weight for 2 h in a conventional oven, and then in a muffle furnace at 450 °C for 3 h.

Lipid Extraction and Fatty Acid Composition Analysis

At the end of each treatment, approximately 1 L of culture was collected and then centrifuged at 4.500 rpm and 14 °C for 15 min. The recovered biomass was frozen at –4.0 °C for 48 h, and then lyophilized for 3-5 days; the dry biomass was stored under refrigeration at 0 °C. Total lipids were extracted following the dry extraction procedure described by Feng *et al.* (2011), and then quantified using the method by Pande *et al.* (1963) using a tripalmitin standard (99%, Sigma-Aldrich).

For the analysis of the fatty acids in the microalgal oil, approximately 10 mg of biomass was mixed with 4 mL of methanol, 2 mL of chloroform, and 0.5 mL of distilled water; the mixture was first sonicated for 15-30 min at 10 °C, and then centrifuged at 4000 rpm for 15 min. The lipid-solvent phase was recovered by washing the upper layer with 2 mL of distilled water, centrifuging and drying with nitrogen-gas. The microalgal lipids were transesterified with 2.5 mL of a HCl:CH_3OH solution (5% v/v) for 2.5 h at 85 °C (Sato and Murata, 1988). The obtained fatty-acid methyl esters (FAMEs) were extracted with 3 mL hexane, washed with distilled water and dried with nitrogen gas, obtaining samples of about 0.5 mL, which were re-suspended in 1 mL of hexane.

FAME profiles were obtained using an Agilent Technology 7890 gas chromatograph (GC). One microliter of the FAME-hexane solution was injected into the GC equipped with a flame ionization detector (FID) and the separation was performed on a DB-23 column (60 m length, 0.32 mm ID, 0.25 μm thickness) with helium as the carrier gas. The temperature of the injector and detector was 250 °C. The temperature program started at 120 °C, holding for 5 min, then increasing at 10 °C.min^{-1} until 180 °C, where it was held for 30 min, and then increased to 210 °C at 10 °C.min^{-1} (held 21 min). A calibration curve was prepared for all FAMEs by injecting known concentrations of an external standard mixture comprising 37 FAMEs (Supelco, Bellefonte, PA, USA), obtaining a correlation coefficient equal to or greater than 95% in all cases.

Calculation of the Specific Growth Rate and Lipid Productivity

The rate of biomass production (or simply the *growth rate*) can be expressed in terms of the specific growth rate μ so that $dX/dt = \mu X$. Since it remains constant during the logarithmic phase:

$$\mu = \frac{\ln(X_2) - \ln(X_1)}{t_2 - t_1} \tag{1}$$

where t_1 and t_2 are cultivation times during the logarithmic phase. The lipid productivity is given by $P_L = d(wX)/dt$; in batch cultures the overall lipid productivity can be approximated by:

$$P_L = \frac{\Delta(wX)}{\Delta t} \tag{2}$$

where $\Delta(wX)$ represents the accumulated lipids from inoculation to harvest, which occurs in the time Δt.

RESULTS AND DISCUSSIONS

In the following sections, the results of the two-stage (TS) cultivation of both strains are first described, followed by the results of the multi-stage (MS) cultivation, and finally the comparison between the two modes.

Effects on the Growth Rate

In the TS mode, *C. vulgaris* exhibited a typical growth curve (Figure 1) in the first stage (C_{90} treatment), starting at nitrogen-rich conditions (90 mg.L^{-1}), increasing the cellular density up to 17.73×10^6 cells.mL^{-1}. In the second stage, after a 50% v/v dilution, and starting at 30, 20, and 10 mg.L^{-1} of N-NH_4 (C_{30}, C_{20}, and C_{10} treatments, respectively), the exponential phase of growth was maintained, but at lower specific growth-rates.

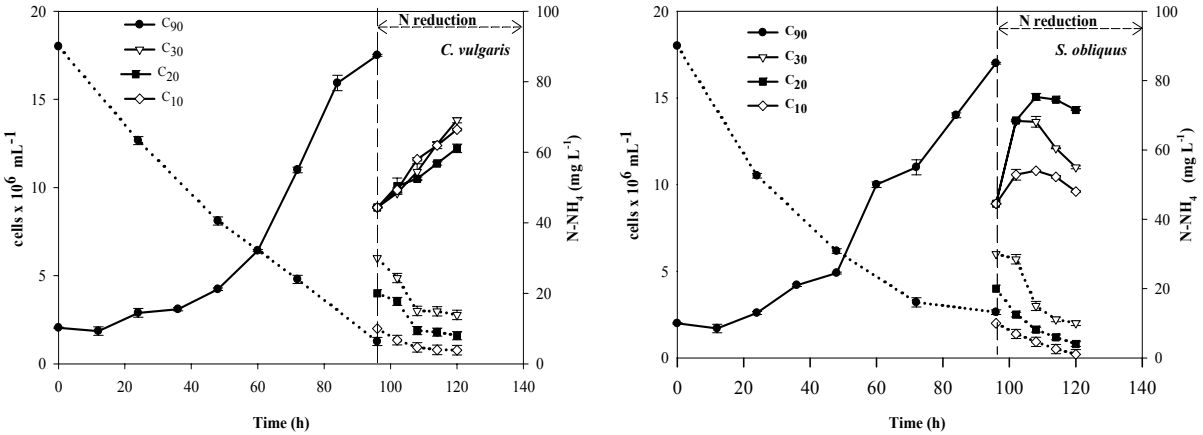

Figure 1: Growth curves (continuous lines) and nitrogen concentration curves (dotted lines) of *S. obliquus* and *C. vulgaris* grown in the TS mode.

A Tukey test showed that the three nitrogen-limited treatments are significantly different ($p \geq 0.05$) from each other, in the maximum cell density achieved and the specific growth rate (Table 2). Although it can be argued that growth limitation was not achieved in this case, the reduced specific growth-rates indicate that the reduction in the available nitrogen did affect the metabolic paths of the culture.

In the case of *S. obliquus*, the nitrogen-rich treatment reached a maximum cell density of 19.9×10^6 cells.mL^{-1}. However, after 12 h into the second stage, the culture entered the stationary phase for all reduced-nitrogen treatments (Figure 1 and Table 2); also, nitrogen uptake continued during the stationary phase, indicating that nitrogen limitation was achieved. Even when *S. obliquus* growth stopped after 12 h into the second stage, the specific growth rate in that period was slower in the C_{20} and C_{10} treatments than in C_{90}. Similar results were reported in microalgae cultures, where the effect of reducing nitrogen through medium dilution caused a reduction in the maximum cell density and the specific growth, leading to accumulation of lipids (Pravenkumar *et al*., 2012;. Mujtaba *et al*., 2012; Blair *et al*., 2014). One reason for this behavior is that certain nutrients (including N, P and Fe ions) are essential for cellular growth and structure synthesis; when these nutrients are present, cellular growth is preferred over lipid synthesis (Ho *et al.,* 2010). However, nitrogen scarcity decreases the synthesis rate of cellular structures (including proteins and nucleic acids), whereas the synthesis rate of carbohydrates and lipids remains steady (Feng *et al.,* 2011; Richardson *et al.,* 1969).

Low concentrations of nitrogen in the medium are associated with low nitrogen-uptake rates per cell. From the data in Table 3, it can be observed that, during the reduced nitrogen stage, *C. vulgaris* consumed ca. 50% of the medium nitrogen, and *S. obliquus* reached a maximum removal percentage of up to 74.2%, suggesting that, even after a sudden reduction of the nitrogen in the medium, both microalgae preserve the potential for nitrogen removal.

The results of MS cultivation of *C. vulgaris* and *S. obliquus* are shown in Figure 2. The maximum cell densities and specific growth rates for this mode of cultivation are shown in Table 4. As expected, the specific growth-rate in both species was higher during the initial nitrogen-rich stage (C_{90}), with values of 0.551 d^{-1} and 0.837 d^{-1} for *C. vulgaris* and *S. obliquus*, respectively, and decreased in the subsequent stages (C_{60}, C_{40}, C_{20}), reacting to the lower nitrogen concentrations in the medium.

Table 2: Maximum cell density and specific growth rates μ in the two-stage mode.

		C. vulgaris		*S. obliquus*	
ID	**Initial N-NH$_4$ concentration (mg L^{-1})**	**Max. cell density (cells×10^6 mL^{-1})**	**μ (d^{-1})**	**Max. cell density (cells×10^6 mL^{-1})**	**μ (d^{-1})**
C_{90}	90	17.73 ± 0.05^{a}	0.694 ± 0.007^{a}	19.90 ± 0.05^{a}	0.638 ± 0.014^{a}
C_{30}	30	13.82 ± 0.14^{b}	0.455 ± 0.008^{b}	14.50 ± 0.05^{b}	$0.600^{\dagger} \pm 0.035^{a}$
C_{20}	20	12.22 ± 0.25^{c}	0.321 ± 0.018^{c}	9.17 ± 0.06^{c}	$0.247^{\dagger} \pm 0.024^{b}$
C_{10}	10	13.29 ± 0.07^{d}	0.397 ± 0.008^{d}	11.93 ± 0.22^{bc}	$0.192^{\dagger} \pm 0.015^{b}$

Different letters in the same column represent significant differences according to Tukey's test ($p \geq 0.05$, $n = 3$), (± Standard deviation).
† Calculated using only two measurements (0 and 12 h). After 12 h the culture reached stationary or death phase.

Table 3: Nitrogen removal, lipid content *w*, and lipid productivity P_L in the TS mode.

Species	Treatment ID	N-NH_4 removal (%)	*w* (%w/w)	P_L (mg L^{-1} d^{-1})	P_L increment† (%)
C. vulgaris	C_{90}	92.96	42.8 ± 6.1[a]	67.24	
	C_{30}	49.96	52.0 ± 5.7[b]	96.17	43.0
	C_{20}	52.84	67.8 ± 1.2[c]	194.92	189.9
	C_{10}	53.31	23.9 ± 2.8[d]	3.00	–95.5
S. obliquus	C_{90}	85.33	37.4 ± 3.2[a]	79.67	
	C_{30}	62.90	60.0 ± 1.4[b]	133.48	67.5
	C_{20}	70.24	63.4 ± 3.3[b]	106.92	34.2
	C_{10}	74.21	24.4 ± 4.6[c]	6.82	–91.4

*Different letters in the same column and for each species represent significant differences according to Tukey's test ($p \geq 0.05$); (± Standard deviation)
† Increment with respect to C_{90}

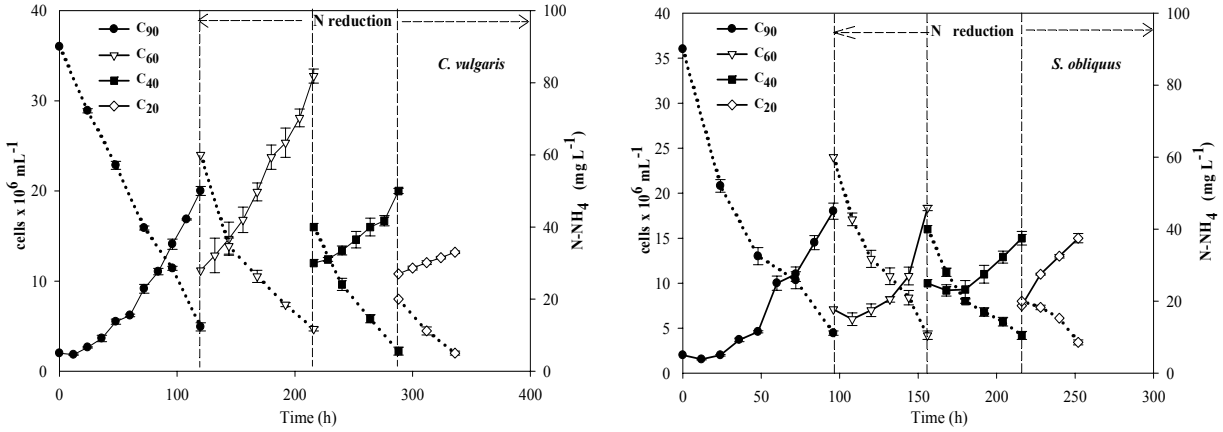

Figure 2: Growth curves (continuous lines) and nitrogen concentration curves (dotted lines) of *S. obliquus* and *C. vulgaris* grown in the MS mode.

Table 4: Maximum cell density and specific growth rates in the MS mode.

		C. vulgaris		*S. obliquus*	
Treatment ID	Initial N-NH_4 concentration (mg L^{-1})	Max. cell density (cells×10^6 mL^{-1})*	μ (d^{-1})	Max. cell density (cells×10^6 ml^{-1})*	μ (d^{-1})
C_{90}	90	22.48 ± 0.53[a]	0.551 ± 0.010[a]	17.27 ± 0.75[a]	0.837 ± 0.027[a]
C_{60}	60	32.75 ± 0.55[b]	0.270 ± 0.004[b]	18.97 ± 0.15[b]	0.360 ± 0.062[b]
C_{40}	40	21.63 ± 0.31[a]	0.134 ± 0.044[c]	15.68 ± 1.83[ab]	0.377 ± 0.127[b]
C_{20}	20	12.17 ± 0.15[c]	0.124 ± 0.013[c]	14.76 ± 1.65[ab]	0.324 ± 0.223[b]

*Different letters in the same column represent significant differences according to Tukey's test ($p \geq 0.05$); (± Standard deviation)

The maximum cell density of *C. vulgaris* remained constant or even increased throughout the MS experiment until the last stage, where it reached a minimum value (Table 4). However, the specific growth-rate did decrease during the first three stages. A similar trend was observed for *S. obliquus* cultures, where the maximum density was achieved in the C_{60} treatment and the specific growth-rate decreased after the second stage. These results show that both species showed a good adaptability to the gradual nitrogen-limitation, since growth did not stop but only slowed down.

It is also noteworthy that in TS cultivation, *S. obliquus* could not adapt to the sudden nitrogen scarcity and almost immediately entered the stationary phase. However, in the MS experiments, the gradual changes in the nitrogen concentrations allowed the microalgae to remain in the exponential phase of growth, with no significant changes in the specific growth rate after the second stage. These results suggest that the growth of both species was limited due to N-reduction in the culture medium, although the effect was stronger in *C. vulgaris*. Measurements of nitrogen removal support this conclusion (see data in Table 5), where the larger percentages of N-removal match the larger values of specific growth rates and *vice versa*.

Table 5: Nitrogen removal, lipid content *w*, and lipid productivity P_L in the MS mode.

Species	Treatment ID	N-NH_4 removal (%)	*w* (%w/w)	P_L (mg L^{-1} d^{-1})	P_L increment† (%)
C. vulgaris	C_{90}	86.31	53.2 ± 3.0[a]	79.19	
	C_{60}	80.40	51.1 ± 2.9[a]	53.46	–32.5
	C_{40}	86.00	54.3 ± 2.8[a]	62.77	–20.7
	C_{20}	74.83	62.9 ± 3.0[b]	108.01	36.4
S. obliquus	C_{90}	87.63	37.6 ± 1.1[a]	60.20	
	C_{60}	82.43	40.1 ± 3.5[a]	77.74	29.1
	C_{40}	73.63	32.4 ± 2.8[ab]	40.06	–33.5
	C_{20}	56.12	28.4 ± 5.9[b]	–19.71	–132.7

*Different letters in the same column and for each species represent significant differences according to Tukey's test ($p \geq 0.05$); (± Standard deviation)
†Increment with respect to C_{90}

Similar observations were reported by Mutlu *et al.* (2011), suggesting that nitrogen-limitation in microalgae cultures causes a decrease in cell density and chlorophyll *a* content, and accumulation of storage lipids. Also, Kim *et al.* (2007) report that *Scenedesmus* sp. exhibited growth inhibition due to the reduction of nitrogen and phosphorous, required by photosynthesis.

Effects on Lipid Content and Productivity

When certain types of stress are induced in the culture, many algae alter their lipid biosynthetic pathways towards the formation and accumulation of fatty acids, which do not perform a structural role, but instead serve primarily as a storage form of carbon and energy (Lin and Lin, 2011). It is generally accepted that stress by nitrogen-limitation inhibits cell division, without an immediate collapse of lipid production (Widjaja *et al*., 2009). In this work, this is well illustrated by the behavior of *S. obliquus* in TS cultivation, in which switching from nitrogen-rich (C_{90}) to nitrogen-limited conditions (C_{30} and C_{20}) caused an increase in lipid content from 37.4% to 60.0% and 63.4% w/w, respectively (Table 3). These values are considerably higher than the 17.7% reported by Gouveia and Oliveira (2009) for the same species in nutrient-sufficient cultures, and similar to those found for *S. obliquus* by Xin *et al.* (2010), of 30% and 50% in cultures with nitrogen and phosphorous limitation, respectively.

The same trend was observed in TS cultivation of *C. vulgaris*, increasing *w* from 42.8% to 52.0% and 67.8% when switching from C_{90} to C_{30} and C_{20}, respectively. These values are considerably higher than those reported by Illman *et al.* (2000) for *C. vulgaris* grown in nutrient-sufficient cultures, ranging from 14 to 30%, and slightly lower than those reported by Rodolfi *et al.* (2009) of 70% w/w under nitrogen-limitation.

In the TS treatments with the largest reduction of nitrogen (C_{90} to C_{10}), the lipid content of both species notoriously decreased. In these cases, *C. vulgaris* remained in the exponential phase of growth, and *S. obliquus* immediately entered the stationary phase. These results suggest that the sudden reduction to 10 mg.L^{-1} of N-NH_4 in the second stage does not allow either species to adjust their metabolism towards lipid storage and growth, which is the result of low adaptability to large nitrogen variations. Xin *et al.* (2010) reported that limiting nitrogen (2.5 mg.L^{-1}) and phosphorous (0.1 mg.L^{-1}) for *Scenedesmus* sp. LX1 caused a considerable increase of the lipid content (30 and 53%, respectively), but the net lipid-productivity is abated by a dominating decrease of the growth rate. Similar conclusions were drawn from the Report of Sheehan *et al.* (1998). However, in their extensive review, Williams and Laurens (2010) identified several works where nitrogen and phosphorous limitation resulted in a net increase of lipid productivity, despite the reduction in growth rates. The results from this work (Table 3) also support this observation: lipid productivities P_L in both species increased when switching from C_{90} to C_{30} and C_{20}, where *C. vulgaris* achieved the highest productivity in the C_{20} batch (194.9 mg.L^{-1}.d^{-1}), and *S. obliquus* in the C_{30} batch (133.5 mg.L^{-1}.d^{-1}). The increase in productivity is associated with lipid content increasing and the growth rate decreasing only moderately. The observed maximum values of P_L are higher than most reported in the literature for photoautotrophic cultures (Table 1), and the trend of increasing P_L as the culture undergoes nitrogen-limitation confirms the trends observed by several previous investigations (Widjaja *et al*., 2009; Griffiths *et al*., 2011; Gouveia and Oliveira, 2009; Ho *et al.,* 2010).

Table 5 shows the results for nitrogen removal, lipid content, and lipid productivity for the MS experiments for both species. In this mode of cultivation,

the effect on lipid content and productivities was different for *C. vulgaris* and *S. obliquus*, supporting the assertion of Griffiths *et al.* (2011) that the response to nitrogen-limitation is species-specific.

For *C. vulgaris*, the second and third stages (C_{60} and C_{40}) did not change the lipid content significantly, and the lipid productivity was reduced from 79.2 to 53.46 $mg.L^{-1}.d^{-1}$ due to the lower growth rate. However, in the fourth stage (C_{20}), both lipid content and productivity increased, achieving a maximum value of 108 $mg.L^{-1}.d^{-1}$, a 36.4% increase relative to the lipid productivity in the first stage. This result is in line with the observations of Widjaja *et al.* (2009), where *C. vulgaris* presented a decrease in lipid productivity after seven days under nitrogen-starvation, but extending the culture for 17 days resulted in a higher lipid productivity. This suggests that *C. vulgaris* needs more than five days of growing under nitrogen-limited conditions to redirect its metabolism towards a significant increase in lipid productivity.

S. obliquus behaved differently in MS cultivation. While in the second and third stages the lipid content did not change significantly, the lipid productivity increased slightly from 60.2 to 77.7 $mg.L^{-1}.d^{-1}$ (Table 5), due to the larger biomass concentration at the end of the second stage. From there, subsequent stages caused a decrease in both lipid content and productivity. From the growth curves of *S. obliquus* under MS cultivation (Figure 2) and the corresponding values of the specific growth rate in Table 4, it is evident that the culture was not greatly affected by the changes of the nitrogen concentration in the cultures, with the exception of the second stage (C_{60}). Thus, *S. obliquus* was able to maintain a steady growth in the exponential phase through the sequential stages, but lipid accumulation by nitrogen-limitation was not observed for this species in the MS mode. This suggests that, under a gradual reduction of nitrogen, *S. obliquus* can use the available nitrogen preferably for cell maintenance, influencing negatively in the lipid accumulation and productivity.

FAMEs Profiles of Microalgal Lipids

When the purpose of lipid production in microalgae cultures is the production of biodiesel, it is important to characterize the TAG fraction for the quality of the resulting biodiesel. Both cultivation time and nitrogen limitation affect microalgal lipids, not only increasing the lipid content, but also changing their composition (Widjaja *et al*., 2009). Thomas *et al.* (1984) analyzed the fatty acid composition of the lipids from seven species of fresh-water microalgae, and observed that all accumulated $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:2,}$ and $C_{18:3}$, that is, a majority of saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA). Hence, for the purpose of biodiesel production it is important to evaluate the changes in the fatty acid profile that may arise from the variations in the cultivation methods.

Tables 6a and 6b summarize the FAMEs profile found in *C. vulgaris* and *S. obliquus*, respectively, under the two cultivation modes studied in this work.

Table 6a: Profile of the fatty acids of *C. vulgaris* lipids (expressed as % of total fatty acids[*]).

	TS[†]			MS			
FAMES	**C_{90}**	**C_{30}**	**C_{20}**	**C_{90}**	**C_{60}**	**C_{40}**	**C_{20}**
C4:0	12.53(0.38)	15.04(1.33)	11.80 (0.59)	9.11 (0.30)	11.24 (0.64)	12.05 (3.25)	13.12 (0.94)
C14:0	10.27 (0.31)	11.36 (0.28)	10.84 (0.34)	8.45 (1.22)	5.47 (0.01)	6.10 (0.27)	6.18 (0.1)
C15:1	n.d.	n.d.	n.d.	8.88 (0.11)	8.03 (0.11)	6.24 (0.06)	6.87 (0.17)
C16:0	7.03 (0.11)	n.d.	7.05 (0.32)	5.12 (0.07)	4.77 (1.09)	3.93 (0.00)	4.15 (0.06)
C16:1	9.75 (0.42)	11.36 (0.28)	9.60 (0.27)	7.07 (0.29)	5.86 (0.13)	6.40 (0.11)	6.26 (0.00)
C17:0	n.d.	n.d.	n.d.	9.13 (0.30)	7.86 (0.04)	6.48 (0.01)	6.97 (0.19)
C18:1n9t	n.d.	n.d.	n.d.	3.99 (0.00)	3.60 (0.04)	6.65 (0.87)	4.93 (0.41)
C20:0	9.48 (0.04)	12.32 (0.17)	10.65 (0.07)	6.85 (0.02)	7.18 (0.22)	8.30 (0.38)	8.33 (0.04)
C18:3n6	n.d.	n.d.	n.d.	n.d.	5.48 (0.09)	4.75 (0.15)	4.52 (0.07)
C20:1	20.79 (0.12)	20.75 (0.12)	20.23 (0.15)	15.08 (0.10)	15.29 (0.46)	14.48 (0.58)	13.73 (0.05)
C21:0	7.17 (0.17)	7.04 (1.26)	7.31 (0.46)	5.20 (0.12)	5.23 (0.18)	4.78 (0.03)	4.87 (0.20)
C22:0	22.97 (0.56)	22.12 (0.58)	22.52 (1.10)	15.64 (0.42)	14.46 (0.36)	15.33 (0.86)	15.20 (0.37)
C22:1n9	n.d.	n.d.	n.d.	5.48 (0.28)	5.52 (0.10)	4.51 (0.03)	4.87 (0.24)
SFA	69.46(0.31)[a]	67.90(0.40)[a]	70.16(0.13)[b]	59.50(0.02)[a]	56.22(0.94)[a]	56.98(1.69)[a]	58.82(0.01)[a]
MUFA	30.54(0.34)[a]	32.11(0.40)[a]	29.83(0.12)[b]	49.50(0.02)[a]	38.30(0.85)[a]	38.27(1.54)[a]	36.66(0.05)[a]
PUFA	n.d.	n.d.	n.d.	n.d.	5.41(0.09)[a]	4.75(0.15)[a]	4.56(0.07)[a]
SFA:MUFA	2.27[a]	2.11[a]	2.35[b]	1.47[a]	1.47[a]	1.49[a]	1.60[a]

[*] Weight percentages, standard deviation between brackets

† Lipid samples in the C_{10} treatment were smaller than the detection limits.

Different letters in the same row represent significant differences according to Tukey's test ($p \geq 0.05$); (± Standard deviation)

n.d. = not detected.

Table 6b: Profile of the FAMEs of *S. obliquus* lipids (expressed as % of total fatty acids*).

	TS†			MS			
FAMES	**C_{90}**	**C_{30}**	**C_{20}**	**C_{90}**	**C_{60}**	**C_{40}**	**C_{20}**
C4:0	12.28 (1.67)	23.85 (1.51)	18.43 (1.55)	11.55 (0.30)	15.50 (0.35)	26.13 (0.54)	21.81 (3.6)
C14:0	11.43 (0.16)	14.74 (1.54)	15.39 (1.62)	9.75 (0.04)	7.72 (0.07)	7.33 (0.06)	7.98 (0.62)
C16:1	10.69 (0.16)	8.54 (0.5)	10.53 (0.26)	9.12 (0.00)	7.61 (0.20)	7.06 (0.01)	6.91 (0.66)
C18:1n9t	n.d.	n.d.	n.d.	4.13 (0.04)	5.10 (0.68)	1.64 (1.08)	5.50 (3.51)
C20:0	14.20 (0.21)	9.19 (0.44)	11.38 (0.31)	12.11 (0.36)	9.59 (0.10)	8.54 (0.04)	8.14 (0.18)
C20:1	20.93 (0.28)	17.71 (2.52)	19.19 (0.90)	17.88 (0.08)	20.73 (0.78)	18.51 (0.07)	17.96 (0.06)
C21:0	n.d.	n.d.	n.d.	7.72 (0.56)	7.39 (0.14)	7.35 (0.01)	6.98 (0.14)
C22:0	20.96 (0.42)	14.09 (0.98)	14.68 (0.42)	17.90 (0.64)	17.32 (0.88)	15.03 (0.05)	16.53 (0.97)
C22:1n9	9.52 (1.64)	11.88 (2.50)	10.40 (1.83)	9.83 (0.90)	9.03 (0.31)	8.42 (0.43)	8.19 (0.47)
SFA	58.87 (1.19)^{a}	61.87 (0.51)^{ab}	59.88 (0.67)^{b}	59.03(0.78)^{a}	57.52(0.41)^{a}	64.37(0.59)^{b}	61.44 (2.32)^{ab}
MUFA	41.13 (1.20)^{a}	38.13 (0.50)^{b}	40.12 (0.66)^{ab}	40.96(0.80)^{a}	42.48(0.42)^{a}	35.62(0.60)^{b}	39.01(2.30)^{ab}
SFA:MUFA	1.43^{a}	1.62^{b}	1.49^{a}	1.44^{a}	1.35^{a}	1.80^{b}	1.60^{ab}

* Weight percentages, standard deviation between brackets

† Lipid samples in the C_{10} treatment were smaller than the detection limits.

Different letters in the same row represent significant differences according to Tukey's test ($p \geq 0.05$); (± Standard deviation)

n.d. = not detected.

It can be observed that the lipids content of *C. vulgaris* is mainly SFA and monounsaturated fatty-acids (MUFA). The profiles show that the ratio of SFAs to MUFAs did not change significantly after reducing nitrogen in the medium, with the exception of the C_{20} treatment, where the production of SFA was slightly favored. Unfortunately, the C_{10} treatments did not yield enough lipids for the GC measurements. On the other hand, cultures of *C. vulgaris* in the MS mode did not exhibit significant changes in the lipid composition throughout the sequential stages, and the overall ratio SFA:MUFA remained constant throughout the sequential batches.

The cultivation mode did affect the FAMEs profile. In the MS mode, the PUFA $C_{18:3}$ was detected for *C. vulgaris*, and the relative compositions of most FAMEs in both species were different than in the TS mode, reaffirming the conclusion by Damiani *et al*. (2010) that the growth conditions affect the FAMEs profile. Similar results were reported by Makulla (2000) for the microalga *S. obliquus*, noting that an increase in the dilution rate caused the SFA fraction to increase from 44.97% in a culture with no dilution to 50.73% when cultivated with a dilution rate of 0.48 d^{-1}. In addition, studies by Lin and Lin (2011) showed that the $C_{18:3}$ fraction decreased dramatically during the nitrogen starvation phase. Finally, Johnson and Wen (2009) found that the C_{16} and C_{18} series of *Chlorella sp*. changed significantly during 5 days of culture.

It is also important to note that the main components of the lipid profiles were long-chain fatty acids ($C_{22:0}$ and $C_{20:1}$), and a large proportion of $C_{4:0}$. This would mean a large cetane number for the resulting biodiesel, although it may result in poor cold-flow properties. Also, the low level of MUFAs and PUFAs suggest that the resulting biodiesel would have good oxidative stability.

In *S. obliquus* lipids, only SFA and MUFA were identified. The main compounds in *S. obliquus* lipids were long-chain fatty acids ($C_{22:0}$ and $C_{20:1}$). Although $C_{4:0}$ concentrations were high (>13% in all treatments), the overall composition was more balanced than in *C. vulgaris* lipids. In the TS mode, the sudden nitrogen limitation had the effect of increasing the composition of lower-sized carbon chains ($C_{4:0}$, $C_{14:0}$), increasing the fraction of SFA, and decreasing the MUFAs content, so that the ratio of SFA:MUFA varied from 1.4-1.6 (in C_{90}) to 2.1-2.3 in C_{30} and C_{20}, respectively. On the other hand, the MS mode did not seem to affect noticeably the fraction of SFA or MUFA, and the ratio SFA:MUFA remained in the range of 1.3 to 1.8.

Similarly to *C. vulgaris*, the composition of *S. obliquus* lipids can lead to good biodiesel quality given a small percentage of MUFAs, virtually no presence of PUFAs, and long-chain fatty acids as main components. Although the latter factor may result in high viscosity, the resulting biodiesel may still be appropriate for concentrated fuel blends.

CONCLUSIONS

Both nitrogen-reduction strategies increased the net lipid-productivity and lipid content. The highest lipid-productivities occurred in TS cultivation (194.92 and 133.48 $mg.L^{-1}.d^{-1}$ for *C. vulgaris* and *S. obliquus*, respectively), and are among the highest values reported to date. *C. vulgaris* adapted well in the TS mode, but in MS cultivation the accumulation of lipids was delayed for at least 5 days. The microalgae

S. obliquus were not able to sustain growth in the TS mode, increasing their lipid content; however, in the MS mode the microalgae adapted well to the reduced nitrogen concentrations and the lipid-accumulation effect was not observed. Both species presented a good potential for nitrogen removal from the artificial wastewater medium, and in general, the removal efficiencies were larger in MS cultivation. The fatty-acid profiles of the lipids from both species showed good potential for biodiesel production in terms of their content of saturated, monounsaturated, and polyunsaturated fatty-acids.

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