

Research Article

Growth, nutrient uptake and chemical composition of *Chlorella* sp. and *Nannochloropsis oculata* under nitrogen starvation

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ABSTRACT. The production of microalgae biomass shows wide valuable uses, in the aquaculture, biotechnology, and food science, among others. However, microalgae show fluctuations in their chemical profile generated mainly by the culture conditions. This study was designated to assess the effects of nitrogen starvation on growth, nutrient uptake, and gross chemical composition of *Chlorella* sp. and *Nannochloropsis oculata*. The control experiments were performed with Conway culture medium in 13-day batch cultures, 12 h photoperiod, and aeration. A second experimental condition was the addition of the nutrients except nitrogen, one week after the start of growth (experiments designed as N-). Cell yield were similar in the control and in the N- experiments for both species. Cell biovolumes did not vary over growth in the control, but both microalgae exhibited larger cell biovolumes in N- experiments, probably as a consequence of the higher accumulation of storage substances. Dissolved nitrogen was exhausted before the end of the experiments, but phosphorus was not totally consumed. Protein and total carotenoid did not vary from the exponential to the stationary growth phase of the control in both species. For *Chlorella* sp., concentrations of lipid did not vary in the control either, but there was a significant increase of carbohydrate over growth. In the N- experiment, concentrations of all substances varied throughout growth of *Chlorella* sp., except lipid. For *N. oculata*, all substances exhibited significant variations over growth, except protein and total carotenoid in the control. Protein and chlorophyll-*a* concentrations decreased over growth in N- experiments for both species. In contrast, concentrations of carbohydrate increased throughout growth in N- experiments, especially in *Chlorella* sp. Nitrogen starvation caused accumulation of carbohydrate, but increments of lipid were restricted to *N. oculata*. Both species showed a fast growth, but the small content of lipid in *Chlorella* sp. is unfavorable for its use as a food-species in a monospecific diet in mariculture, and as a feedstock for biodiesel production. *N. oculata* is a lipid-rich species, and its lipid content can be successfully incremented through nitrogen starvation. This species is promising in uses that demand high concentrations of lipid, such as the production of biodiesel.

Keywords: biodiesel, biomass, cultivation, lipid, mariculture, productivity.

Crecimiento, absorción de nutrientes y composición química de *Chlorella* sp. y *Nannochloropsis oculata* bajo carencia de nitrógeno

RESUMEN. La producción de biomasa de microalgas presenta variados y valiosos usos en la acuicultura, biotecnología y ciencias de los alimentos, entre otros. Sin embargo, las microalgas muestran fluctuaciones en su perfil químico causado principalmente por las condiciones de cultivo. En este estudio se evaluó los efectos de la carencia de nitrógeno sobre el crecimiento, absorción de nutrientes y composición química bruta de *Chlorella* sp. y *Nannochloropsis oculata*. Los experimentos control se efectuaron en un medio de cultivo Conway durante 13 días, 12 h de fotoperiodo y aeración. Una segunda condición experimental fue la adición de nutrientes, excepto nitrógeno, una semana después del inicio del crecimiento (denominados experimentos N-). Los rendimientos celulares fueron similares en el control y en los experimentos N- en ambas especies. Los biovolúmenes celulares no variaron con el crecimiento en el control, pero ambas microalgas presentaron mayores biovolúmenes celulares en los experimentos N-, probablemente como consecuencia de una mayor acumulación de sustancias almacenadas. El nitrógeno disuelto se agotó antes del término de los experimentos, pero el fósforo no fue totalmente consumido. En ambas especies, las proteínas y carotenoides totales no variaron

desde la fase exponencial hasta la fase estacionaria de crecimiento del control. En *Chlorella* sp., las concentraciones de lípidos no variaron en el control, pero se determinó un aumento significativo de carbohidratos durante el crecimiento. En los experimentos N-, las concentraciones de todas las sustancias variaron durante el crecimiento de *Chlorella* sp., a excepción de los lípidos. En *N. oculata*, todas las sustancias presentaron variaciones significativas durante el crecimiento, excepto proteínas y carotenoides totales en el control. Las concentraciones de proteínas y clorofila-*a* disminuyeron durante el crecimiento en los experimentos N- de ambas especies. En contraste, las concentraciones de carbohidratos aumentaron durante el crecimiento en los experimentos N-, especialmente en *Chlorella* sp. La carencia de nitrógeno causó una acumulación de carbohidratos, pero el incremento de lípidos se restringió a *N. oculata*. Ambas especies presentaron un crecimiento rápido, pero el escaso contenido de lípidos en *Chlorella* sp. es desfavorable para su uso como alimento en una dieta mono-específica en acuicultura y como materia prima para la producción de biodiesel. *N. oculata* es una especie rica en lípidos y su contenido en lípidos puede ser incrementado exitosamente a través de la carencia de nitrógeno. Esta especie es promisoría en usos que demanden altas concentraciones de lípidos, como la producción de biodiesel.

Palabras clave: biodiesel, biomasa, cultivo, lípidos, maricultura, productividad.

INTRODUCTION

Microalgae are the main components of the phytoplankton, and thus they are the most important primary producers of the majority of aquatic systems (Falkowski & Raven, 2007). The term phytoplankton encompasses a heterogeneous set of microscopic eukaryotic lineages, resulting in a broad diversity of not related groups such as green algae, diatoms, dinoflagellates, and euglenoids, among others (Graham *et al.*, 2009). Cyanobacteria are prokaryotic, but they are also included in the phytoplankton due their ecological and evolutionary role as primary producers (Ratti *et al.*, 2013). Marine phytoplankton is often categorized into groups based on taxonomic traits, abundance, role in biogeochemical fluxes, and/or primary production. While diatoms are considered the principal group contributing to primary production and carbon export in coastal areas, dinoflagellates are important contributors to biomass in stratified or silica-limited areas, and cyanobacteria are the dominant group in offshore continental shelf and oceanic waters (Silva *et al.*, 2009).

Many lineages of microalgae include fast-growing microorganisms with very high growth rates under optimum culture conditions. Fast-growing characteristics combined with a huge chemical diversity open possible applications of microalgae biomass in many fields, such as aquaculture, biotechnology, and food science (Spolaore *et al.*, 2006; Templeton & Laurens, 2015), for instance. It is expected an increase and a diversification of applications involving microalgae, as a consequence of the ongoing search for more productive systems to supply the society with food, feedstocks, and high value biochemical products (Zeng *et al.*, 2011; Lee-Chang *et al.*, 2013). Despite microalgae biotechnology is still in its childhood, its development has been done in a context of sustainable production using modern and efficient processes (Wijffels *et al.*, 2013). Particularly in food science,

microalgae are useful to improve the nutritional content of conventional foods and hence to positively affect human health, due to their favorable chemical composition (Tokuşoglu & Ünal, 2003). In recent years, a growing attention on microalgae focuses on the possible use of the biomass as a feedstock for biofuel production (Van Iersel & Flammini, 2010; Zeng *et al.*, 2011; Mubarak *et al.*, 2015).

Despite the wide scenario of possible applications, microalgae advantages and drawbacks should be considered without excessive enthusiasm, but exclusively with a scientific approach (Van Iersel & Flammini, 2010). There are both technological and biological issues to achieve a broader use of them. Regarding the technological view, it is necessary to develop more efficient systems to produce biomass and valuable microalgae-based products (Wijffels & Barbosa, 2010). On the other hand, biological issues include the domestication of promising strains (Lim *et al.*, 2012), and the successful uses of mechanisms to stimulate microalgae to grow and produce target substances (Kaye *et al.*, 2015). All possible applications of microalgae are directly coupled to high growth and a favourable chemical profile of the species (Borges-Campos *et al.*, 2010). Fluctuations in the chemical profile of microalgae in cultures are a key issue in their study and applications (Lourenço *et al.*, 2002).

The chemical content of microalgae can vary with culture age and with changes in culture conditions (Carvalho *et al.*, 2009). The effect of variation of culture parameters on many microalgae species has been studied in order to better understand their physiology, as well as to answer specific and relevant questions for mass culture (Grobbelaar, 2014). Data on the chemical composition of microalgae may also vary widely due to differences of the methods of measurement used (Barbarino & Lourenço, 2005), the physiological state of the microalgae (Geider & La Roche,

2002), as well as to the experimental conditions applied, like temperature (Durmaz *et al.*, 2009), light intensity (Lourenço *et al.*, 2008), and culture medium (Huerlimann *et al.*, 2010), especially in batch cultures. Due to the interaction of the organisms with the culture medium, a batch culture is under continuous chemical change. These variations reflect on the cell metabolism and consequently on their chemical composition (Lourenço *et al.*, 2002). Thus, the chemical composition of a given species may vary widely under different growth conditions, and such changes may be related to the growth phase of the culture (Costard *et al.*, 2012). However, studies focusing sampling in different growth phases are relatively scarce; most of the papers report the chemical profile of given species in a fixed momentum of the cultivation, ignoring the continuing process of interaction between microalgae and the medium.

Nitrogen is added to most culture media in high concentrations, and changes in N supply are known to influence strongly both growth and chemical composition of microalgae (Valenzuela-Espinoza *et al.*, 1999; Harrison & Berges, 2005). Studies of the effects of nitrogen sources on the chemical composition in different growth phases may give important information on species metabolism (Fidalgo *et al.*, 1995). On the other hand, this knowledge is also important for both aquaculture activities and biotechnological applications, in which different culture media (with nutrients in various chemical forms) may be used (Sepúlveda *et al.*, 2015). Under many culture conditions, microalgae may experience nitrogen starvation at least in part of their growth, especially in the stationary growth phase of batch cultures (Lourenço *et al.*, 2004). Photosynthetic activity and nitrogen metabolism are processes integrally coupled, and a nitrogen limitation affects the photosynthesis in microalgae by reducing the efficiency of light collection due a decline in cell pigment content (Geider *et al.*, 1993). This physiological stress may promote strong effects on the chemical composition of a given microalga, such as demonstrated by several authors (*e.g.*, Silva *et al.*, 2009; Jiang *et al.*, 2011; Urreta *et al.*, 2014). Production and accumulation of protein, carbohydrate, lipid, and carotenoids are of particular importance if the microalgae are cultivated either to feed marine animals or to produce specific valuable substances, for instance (Machado & Lourenço, 2008).

This paper aimed to assess growth, nutrient uptake, and chemical composition of two marine microalgae, the trebouxiophycean *Chlorella* sp. and the eustigmatophycean *Nannochloropsis oculata* under standardized culture conditions and under nitrogen starvation. Growth under nitrogen starvation is thought to induce

protein and chlorophyll decreases, and increments in carbohydrate and lipid productivity of microalgae, but the intensity of these processes vary widely depending on the species tested and experimental conditions. The effects of the culture conditions on the two microalgae were discussed in the context of possible use of the microalgae as food-species in mariculture and as feedstocks for biofuel production.

MATERIALS AND METHODS

The microalgae tested

Two strains were used in this study: *Chlorella* sp. (division Chlorophyta, class Trebouxiophyceae, strain CMEA BS04), and *Nannochloropsis oculata* (division Heterokontophyta, class Eustigmatophyceae, strain CMEA MO08). Both strains are available at Elizabeth Aidar Microalgae Culture Collection, Fluminense Federal University, Brazil (Lourenço & Vieira, 2004).

Culture conditions

Starter cultures of 150-250 mL in mid-exponential growth phase were inoculated into 5 L of seawater, previously autoclaved at 121°C for 60 min in 6-L borosilicate flasks. The microalgae were cultured in two experimental conditions:

a) Seawater enriched with Conway nutrient solutions (Walne, 1966) in its original concentrations and continuously bubbled with filtered air at a rate of 2 L min⁻¹. This experimental condition is designed as “control”.

b) The same conditions described in item *a*, including a new addition of Conway nutrient solutions without nitrogen in the 7th day of growth. This procedure promoted the enrichment of the culture medium with the theoretical concentrations of all elements of the Conway's receipt (*e.g.*, 128 μM NaH₂PO₄·H₂O, 1.82 μM MnCl₂·4H₂O, 4.81 μM FeCl₃·6H₂O, etc.), except NaNO₃. The actual concentrations of the chemical components available to the microalgae from the 7th day of cultivation corresponded to the sum of the enrichment done plus the residual concentrations of the original substances that had not been taken up yet. This experimental condition is designed as “N⁻”.

Each experiment was carried out in four culture flasks (n = 4), exposed to 350 μmol photons m⁻² s⁻¹ measured with a Biospherical Instruments Quanta Meter, model QLS100, provided from beneath by 40 W fluorescent lamps (Sylvania daylight tubes), on a 12:12 h light:dark cycle. Mean temperatures in experiments were 21 ± 1°C and the salinity was 33.0. Growth was estimated based on daily microscopic cell counting with Neubauer chambers. Cultures were not buffered

and pH was determined daily, at the beginning (15-30 min after the start of the photoperiod), in the middle (6 h after the start of the photoperiod), and in the end of the light period (11 h after the start of the photoperiod). All samplings for cell counts occurred during the first 30 min of the light period. The initial cell densities of cultures were 3.0×10^4 cell mL⁻¹ for *Chlorella* sp. and 2.5×10^5 cell mL⁻¹ for *N. oculata*. The experiments were carried out for 13 days. Growth rates were calculated daily using the following equation:

$$r = \frac{\ln N_t - \ln N_{t-1}}{\Delta t}$$

where r is the growth rate; \ln is the logarithm of the number of cells in a given day, in a standardized time (beginning of the photoperiod), calculated using the basis 2; N_t is the number of cells recorded in a given day (t); N_{t-1} is the number of cells 24 h before the counting carried out in the time t (in days); Δt is the difference, in days, between the two cell countings.

The biovolumes of the cells were measured using the equation provided by Hillebrand *et al.* (1999), assuming a spherical shape for both microalgae. Mean volumes were based on measurements of 30 cells in each culture flask, giving four mean values used for statistics ($n = 4$).

Sampling procedure

Each culture was sampled twice for chemical analysis (on the 7 and 13th days of growth), corresponding to late-exponential and stationary growth phases. Samples of 1.6 to 2.3 L were concentrated by centrifugation at $8,000 \times g$ for 9.0 min, at least once, using a Sigma centrifuge, model σ -15, to obtain highly concentrated pellets. Before the last centrifugation, cells were washed with artificial seawater (Kester *et al.*, 1967) prepared without nitrogen, phosphorus and vitamins, and adjusted to salinity of 10. All supernatants obtained for each sample were combined and the number of cells was determined (using Neubauer chambers) to quantify possible cell losses. The pellets were frozen at -18°C and then freeze-dried (using a Terroni-Fauvel, model LB 1500 device), weighed and stored in desiccators under vacuum and protected from light until the chemical analyses. Samples to be analyzed for chlorophyll and carotenoid were obtained by filtering the cultures under vacuum onto Whatman GF/F[®] glass microfibre filters (0.7 μm nominal pore size). The filtered samples were kept at -18°C in flasks containing silica-gel until analysis. All sampling for chemical analysis was done during the first 90 min of the light period.

Daily samples of the culture medium were taken to evaluate the uptake of dissolved nutrients by the

microalgae. At the first 30 min of the photoperiod, samples of 40 to 60 mL of the cultures from each flask were collected. The samples were filtered in the same manner as described above for photosynthetic pigments. The filtered samples of culture media were kept at -18°C in polyethylene flasks until analysis of dissolved nutrients.

Chemical analysis

Total nitrogen and phosphorus were determined by peroxymonosulfuric acid digestion, using a Hach digester (Digesdhal[®], Hach Co., model 23.130-20) (Hach *et al.*, 1987). Calibration curves were prepared using NH_4Cl and NaHPO_4 as standards of nitrogen and phosphorus, respectively. See Lourenço *et al.* (2005) for further details.

The Lowry *et al.* (1951) method was used to evaluate protein in the samples, with bovine serum albumin as a protein standard, following the extraction procedures proposed by Barbarino & Lourenço (2005). Spectrophotometric determinations were done at 750 nm, 35 min after the start of the chemical reaction. Total carbohydrate was extracted with 80% H_2SO_4 , according to Myklestad & Haug (1972). The carbohydrate concentration was determined spectrophotometrically at 485 nm, 30 min after the start of the chemical reaction, by the phenol-sulfuric acid method (Dubois *et al.*, 1956), using glucose as a standard. Total lipid was extracted following Folch *et al.* (1957), and determined gravimetrically after solvent evaporation.

Chlorophyll-*a* and carotenoid were extracted in 90% acetone at 4°C for 20 h, after grinding the filters with the samples. Spectrophotometric determination of chlorophyll-*a* was carried out as described by Jeffrey & Humphrey (1975), and the determination of total carotenoid was carried out as described by Strickland & Parsons (1968).

Determinations of ammonia + ammonium followed the procedure proposed by Aminot & Chaussepied (1983), nitrite and nitrate analyses were performed following Strickland & Parsons (1968), and phosphate was determined according to Grasshoff *et al.* (1983). All nutrients were analyzed spectrophotometrically.

Statistical analysis

The results of growth and chemical composition were compared using Student's *t*-test (Zar, 1996), adopting a level of significance $\alpha = 0.05$.

All experiments were performed twice in order to confirm the results. The chemical analyses were also done twice, using samples generated by all experiments carried out. In this paper, we show the results of only one set of experiments.

RESULTS

Growth curves of *Chlorella* sp. and *Nannochloropsis oculata* in the two experiments are shown in Figures 1 and 2, respectively. For both species, final cell yields were similar in the control and in the treatment with nitrogen starvation ($P > 0.05$). Cell densities at the end of the experiments were 6.43×10^6 cell mL⁻¹ (control) and 5.97×10^6 cell mL⁻¹ (N-) for *Chlorella* sp., and 6.96×10^7 cell mL⁻¹ (control) and 6.52×10^7 cell mL⁻¹ (N-) for *N. oculata*. For each species, similar growth rates were found in the experiments throughout the exponential growth phase. For *Chlorella* sp., growth rates fluctuated around 1.0-1.1 (with basis on log₂ of cell numbers) throughout the exponential growth phase, which generated increases of 2.0 to 2.3-fold of cell densities every 24 h. In the stationary growth phase rates were lower (typically <0.15). For *N. oculata*, growth rates fluctuated around 1.2-1.3 (with basis on log₂ of cell numbers) throughout the exponential growth phase, which generated increases of 2.3 to 2.7 fold of cell densities every 24 h. In the stationary growth phase rates were lower (typically <0.10), with similar values for the control and the N- experiment.

Cell biovolumes of both species did not vary throughout the exponential growth phases of all experiments ($P > 0.23$, Figures 3-4). However, in the stationary growth phase significant differences were found, with larger cell biovolumes in N- treatment ($P < 0.01$, Figures 3-4). *Chlorella* sp. exhibited no significant variations in cell biovolumes in the control, with average values around $61.5 \mu\text{m}^3$ throughout the experiment. Similar values were found for *Chlorella* sp. in the exponential growth phase of the N- treatment, but increases in cell biovolumes were detected throughout the stationary growth phase, achieving a peak of $88.7 \mu\text{m}^3$ in the last day of cultivation. Similarly, *N. oculata* did not show variations in cell biovolumes in the control, with an average value of $14.1 \mu\text{m}^3$. In the N-treatment, cell biovolumes were smaller in the exponential growth phase (average of $14.0 \mu\text{m}^3$), increasing throughout the stationary growth phase to achieve $18.7 \mu\text{m}^3$ in the 13th day of cultivation.

In both control and N- experiments, pH values fluctuated widely throughout the photoperiod (data not shown). Measurements of pH at the start of the photoperiod gave always lower values, typically ca. 8.0. The values of pH increased throughout the photoperiod and achieved ~8.9 after 11 h of light in the second half of the exponential growth phase (from day 3 to day 7 of growth). In the stationary growth phase (from the 9th day of growth) daily maximum values of

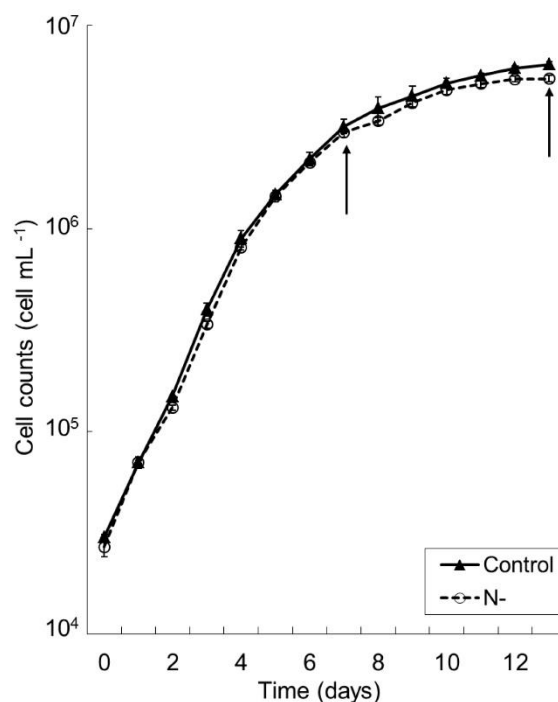


Figure 1. Growth curves of *Chlorella* sp. cultured in two different experimental treatments: standardized conditions (control) and under nitrogen limitation from the 7th day of growth (N-). Arrows indicate the moments in which cells were sampled to perform chemical analyses. Each point in the curves represent the mean of four replicates \pm standard deviation ($n = 4$).

pH were lower (typically <8.8) than in the exponential phase.

There was a remarkable trend of decreasing the concentrations of dissolved nitrate and phosphate throughout the experiments (Figs. 5-6). Nitrate depletion occurred in the 10 or 11th day of cultivation in all experiments. Phosphate uptake was almost total in the control for *N. oculata*, achieving $1.3 \mu\text{M}$ at the 13th day of cultivation (Fig. 6). For *Chlorella* sp., in the control the average phosphate concentration was $9.4 \mu\text{M}$ at the 13th day of cultivation, which is equivalent to ~92.7% consumption of the initial concentration of phosphate added to the culture medium (Fig. 5). In the experiments N- the enrichment with phosphate occurred when the cultures still had some 27-34 μM of the original dissolved phosphate, generating peaks of phosphate (~155 μM) in 7th day of growth, as a result of the addition of Conway nutrient solutions without NaNO_3 . A high concentration of phosphate was still present in the culture medium at the end of the N-experiments: 73 μM for *Chlorella* sp. and 81 μM for *N. oculata*. Variable concentrations of nitrite (0.0-29.6 μM) and ammonia/ammonium (0.0-3.7 μM) were also

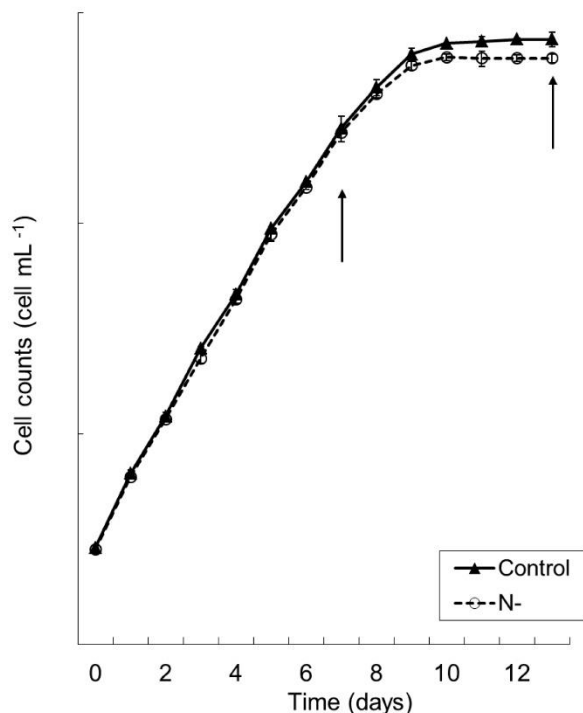


Figure 2. Growth curves of *Nannochloropsis oculata* cultured in two different experimental treatments: standardized conditions (control) and under nitrogen limitation from the 7th day of growth (N-). Arrows indicate the moments in which cells were sampled to perform chemical analyses. Each point in the curves represent the mean of four replicates \pm standard deviation ($n = 4$).

detected in all experiments throughout growth (Figs. 5-6). Nitrite concentrations tended to increase from the beginning to the end of the exponential growth phase, progressively decreasing until the end of the stationary growth phase. Variations in the ammonia/ammonium concentrations did not show a clear pattern in the experiments, with high fluctuations throughout growth, but the values were always negligible in comparison to the nitrate concentrations.

Total concentration of nitrogen and phosphorus in cells of *Chlorella* sp. and *N. oculata* are showed in Table 1. Results refer to the stationary growth phase only. For both species, total nitrogen concentrations were higher in the control. Conversely, total phosphorus concentrations were significantly higher at the end of the N- experiments.

Values for protein, carbohydrate, lipid, chlorophyll-*a*, and total carotenoid are shown in Tables 2 and 3. The protein content did not vary in the control experiments for both species ($P > 0.05$). However, the protein content decreased significantly from the exponential to the stationary growth phase for both microalgae cultured in the N- experiments ($P < 0.01$). The

carbohydrate content increased throughout growth of both species in all treatments. In *Chlorella* sp., increments of the carbohydrate content from the exponential to the stationary growth phase were more intense than in *N. oculata* (Tables 2 and 3), with a peak concentration of 54.5% of the dry matter (d.m.) in the N- experiment. There was no variation in the lipid content of *Chlorella* sp. in different treatments and growth phases, with values fluctuating around 14.5% d.m. ($P > 0.29$). On the other hand, significant variations occurred in the lipid content of *N. oculata* in both treatments and growth phases ($P < 0.01$), with increases of the lipid content from the exponential to the stationary growth phase (Table 3). The highest concentration of total lipid was found in *N. oculata* in the N- experiment (33.7% d.m.). Chlorophyll-*a* concentrations decreased for both species in all experiments from the exponential to the stationary growth phase ($P < 0.05$). *Chlorella* sp. tended to show higher values for chlorophyll-*a* than *N. oculata*. No variations in total carotenoid content were found in the control experiments for both species ($P > 0.19$). However, in the N- experiments of both species, total carotenoid content increased from the exponential to the stationary growth phase ($P < 0.01$). Absolute concentrations of total carotenoid tended to be higher in *Chlorella* sp. than in *N. oculata*.

DISCUSSION

Growth, nutrient consumption, and cell biovolumes

Chlorella sp. and *Nannochloropsis oculata* exhibited similar growth curves in the treatments tested here. The chemical composition of the culture medium and the input of carbon in the experimental flasks are two of the main factors that influence the growth of microalgae in cultures (Wood *et al.*, 2005). Current experiments were performed using the Conway culture medium, which shows a nutrient-rich composition with 1.18 mM of nitrogen and 128 μ M of phosphorus, besides high concentrations of trace-metals and other components. In this context, the depletion of nutrients is unlikely to occur quickly in comparison to what typically occurs with other culture media, such as the f/2 culture medium (Guillard, 1975). By comparison, the f/2 culture medium shows only 880 μ M of nitrogen and 36 μ M of phosphorus. The availability of higher concentrations of nutrients tends to generate a faster growth, especially if the cultures are supplied with carbon sources. Our experiments were run with the addition of constant aeration, which promotes the input of CO₂ in the culture medium. In aerated cultures a constant input of carbon is obtained by dissolving more CO₂ from the air into the culture medium. Considering

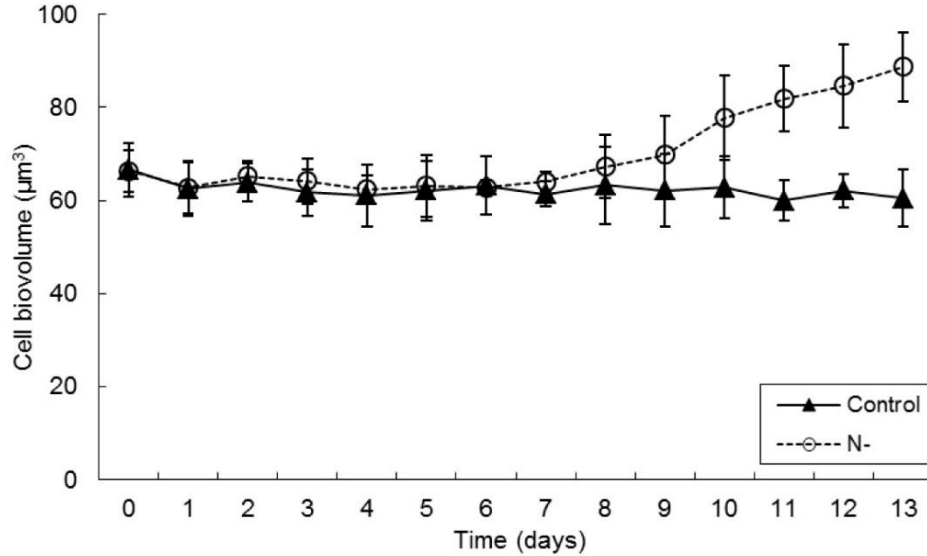


Figure 3. Measurements of cell biovolumes of *Chlorella* sp. cultured in two different experimental treatments: standardized conditions (control) and under nitrogen limitation from the 7th day of growth (N-). Values are expressed as μm^3 , and represent the mean of four replicates \pm standard deviation ($n = 4$).

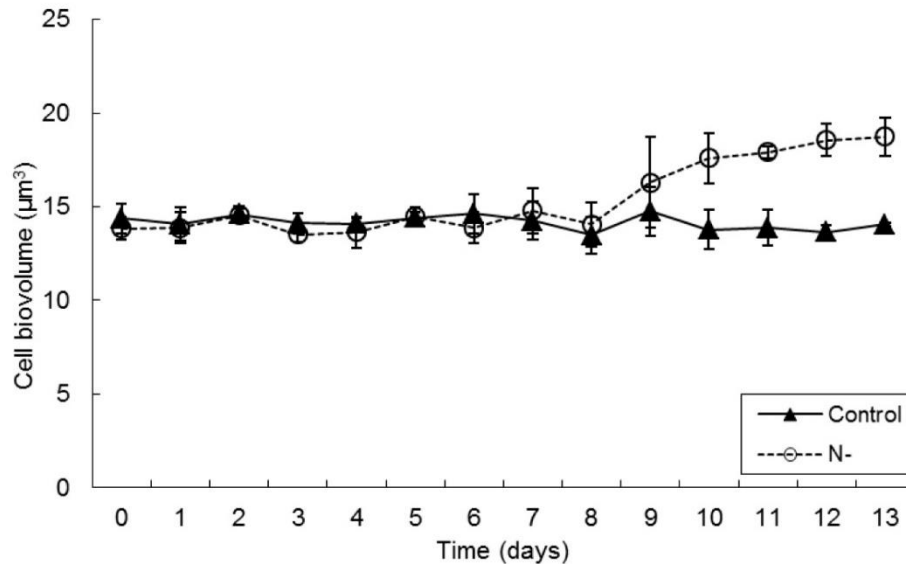


Figure 4. Measurements of cell biovolumes of *Nannochloropsis oculata* cultured in two different experimental treatments: standardized conditions (control) and under nitrogen limitation from the 7th day of growth (N-). Values are expressed as μm^3 , and represent the mean of four replicates \pm standard deviation ($n = 4$).

the coupling of carbon and nitrogen metabolism, as demonstrated by Turpin (1991), the availability of carbon should make the assimilation of nitrogen faster, supplying cells with carbon for amino acid synthesis. As a consequence, aerated cultures tend to run out of nitrogen faster than non-aerated cultures, especially in

the stationary growth phase of batch cultures (Lavín & Lourenço, 2005). The faster assimilation of nitrogen, coupled to the greater availability of carbon, is probably the main factor to promote higher final cell yields in aerated cultures. Previous experiments performed by Lourenço *et al.* (2004) with 12 algal species, including

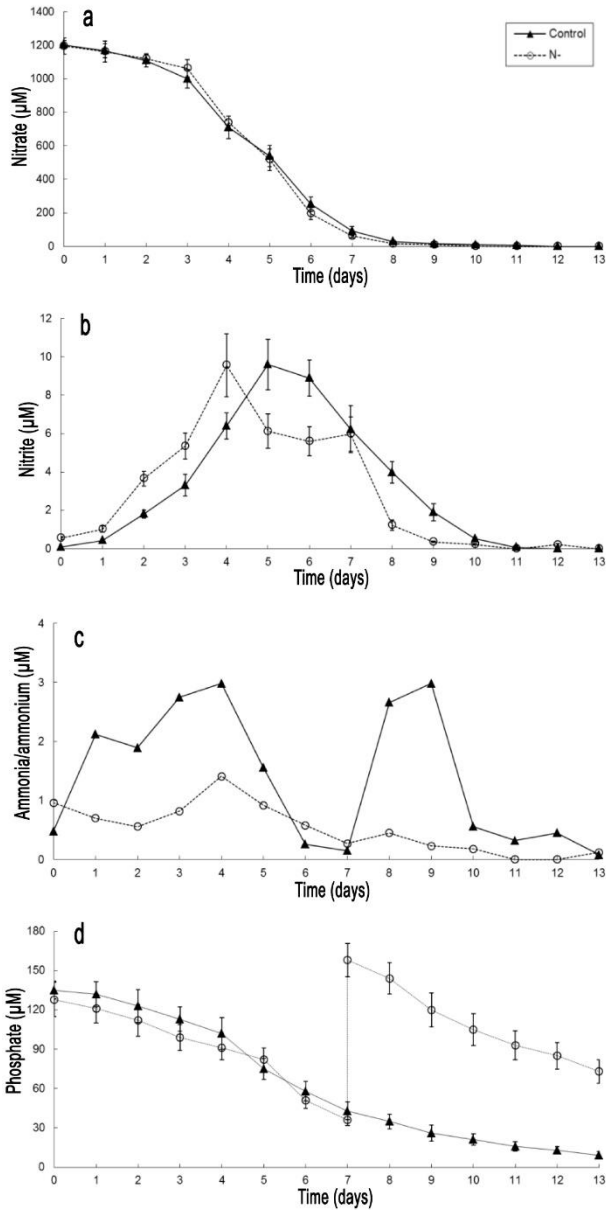


Figure 5. Variations of the a) nitrate, b) nitrite, c) ammonia/ammonium, and d) phosphate concentrations throughout the growth of *Chlorella* sp. in two experimental treatments: standardized conditions (control) and under nitrogen limitation from the 7th day of growth (N-). Each point in the curves represent the mean of four replicates \pm standard deviation (n = 4).

Chlorella minutissima and *Nannochloropsis oculata*, recorded evidence of carbon limitation in all cultures run without inputs of aeration. For the 12 species tested, there was a significant greater final yield of microalgae cultured with aeration in comparison to the treatment without aeration.

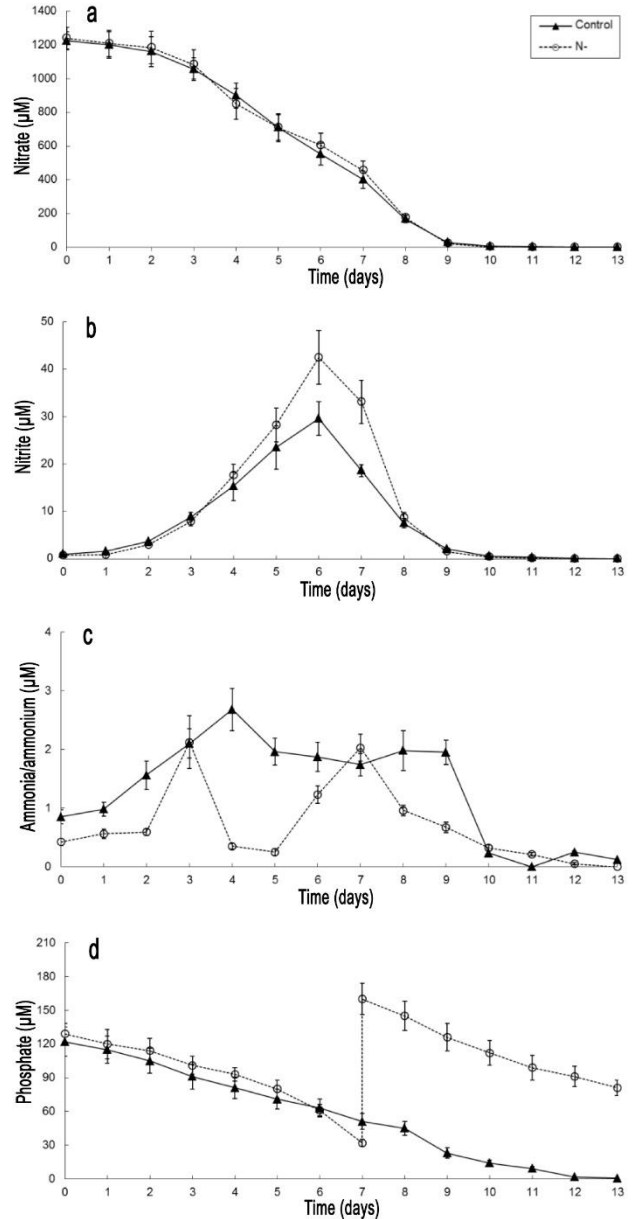


Figure 6. Variations of the a) nitrate, b) nitrite, c) ammonia/ammonium, and d) phosphate concentrations throughout the growth of *Nannochloropsis oculata* in two experimental treatments: standardized conditions (control) and under nitrogen limitation from the 7th day of growth (N-). Each point in the curves represent the mean of four replicates \pm standard deviation (n = 4).

Some authors suggest that the use of pure CO_2 is a more effective condition to increase microalgae biomass in nutrient-rich culture media. This kind of recommendation is particularly common in studies on biotechnological uses of the biomass, in which it is necessary to achieve high productivities (Eriksen *et al.*,

Table 1. Total concentrations of intracellular nitrogen and phosphorus of the microalgae *Chlorella* sp. and *Nannochloropsis oculata* in the stationary growth phase of two different experimental treatments: standardized conditions (control) and under nitrogen limitation from the 7th day of growth (N-). Values are expressed as percentages of substances in relation to the dry matter. Each value represents the mean of four replicates \pm standard deviation (n = 4).

Microalga/experiment	Total nitrogen	Total phosphorus
<i>Chlorella</i> sp.	***	**
Control	6.16 \pm 0.12 ^a	0.88 \pm 0.06 ^b
N-	3.69 \pm 0.13 ^b	1.29 \pm 0.11 ^a
<i>N. oculata</i>	***	**
Control	5.22 \pm 0.31 ^a	0.91 \pm 0.10 ^b
N-	3.70 \pm 0.18 ^b	1.37 \pm 0.12 ^a

§ Mean values significantly different: ** $P < 0.01$, a > b; *** $P < 0.001$, a > b.

Table 2. Chemical profile of the trebouxiophycean *Chlorella* sp. in two growth phases and two different experimental treatments: standardized conditions (control) and under nitrogen limitation from the 7th day of growth (N-). Values are expressed as percentages of substances in relation to the dry matter. Each value represents the mean of four replicates \pm standard deviation (n = 4).

Experiment/growth phase	Protein	Carbohydrate	Lipid	Chlorophyll- <i>a</i>	Total carotenoid
Control		***		*	
Exponential phase	34.6 \pm 3.8	23.3 \pm 2.5 ^b	13.6 \pm 0.9	1.05 \pm 0.06 ^a	0.41 \pm 0.04
Stationary phase	27.7 \pm 2.9	40.5 \pm 4.1 ^a	14.9 \pm 1.1	0.84 \pm 0.05 ^b	0.48 \pm 0.04
N-	**	***		*	**
Exponential phase	35.8 \pm 2.3 ^a	22.6 \pm 2.0 ^b	13.9 \pm 0.9	1.01 \pm 0.08 ^a	0.41 \pm 0.05 ^b
Stationary phase	16.6 \pm 1.2 ^b	54.5 \pm 4.6 ^a	15.3 \pm 1.0	0.72 \pm 0.07 ^b	0.60 \pm 0.06 ^a

Mean values significantly different: * $P < 0.05$, a > b; ** $P < 0.01$, a > b; *** $P < 0.001$, a > b. The absence of letters indicates that mean values are not significantly different.

1998; Doucha *et al.*, 2005; Grobbelaar, 2014; Maity *et al.*, 2014). However, significant increases of microalgae biomass are not always detected as a consequence of the input of pure CO₂ in the medium. For instance, Faria *et al.* (2012) run experiments with the marine eustigmatophycean *Nannochloropsis oculata* with input of pure CO₂ and the excess of dissolved carbon did not promote a greater growth in comparison to the simple and continuous aeration (such as done here). Similarly, Chiu *et al.* (2009) found the highest productivity with cultures of *N. oculata* with a small enrichment with carbon and either neutral or negative effects on productivity in treatments with high concentrations of pure CO₂. The absence of increments of growth as a response to the input of pure CO₂ may result of other limiting factors, such as irradiance or pH imbalance (MacIntyre & Cullen, 2005).

Daily variations of pH values in the experiments were relatively small (from 8.0 to 8.9), considering that the cultures were not buffered (Sunda *et al.*, 2005). The typical low values of pH at the start of the photoperiod are derived from the continuous aeration in the dark period without photosynthetic activity, promoting CO₂

inputs that lowered the pH values (Goldman *et al.*, 1982). Active photosynthesis and uptake of dissolved carbon by the cells throughout the photoperiod resulted in increments of the pH values, which achieved up to 8.9 in the exponential growth phase and up to 8.8 in the stationary growth phase. Wider variations of pH in the exponential growth phase are explained by a more intense photosynthetic activity (MacIntyre & Cullen, 2005). Although in the stationary growth phase the cell numbers were higher, a less intense photosynthetic activity due to nitrogen limitation generated less uptake of dissolved carbon from the medium, which promoted smaller variations in pH throughout the photoperiod. Variations in pH values of non-buffered cultures are strongly influenced by the metabolic activities of the microalgae, especially by the photosynthesis (Goldman *et al.*, 1982).

The analysis of Figures 1 and 2 shows that after one week of growth, the nitrogen availability was equivalent to less than 20% of the initial concentrations supplied by Conway stock solutions. Considering the high cell numbers at this moment, it is coherent to suggest that there was a nitrogen limitation for growth.

Table 3. Chemical profile of the eustigmatophycean *Nannochloropsis oculata* in two growth phases and two different experimental treatments: standardized conditions (control) and under nitrogen limitation from the 7th day of growth (N-). Values are expressed as percentages of substances in relation to the dry matter. Each value represents the mean of four replicates \pm standard deviation (n = 4).

Experiment/growth phase	Protein	Carbohydrate	Lipid	Chlorophyll- <i>a</i>	Total carotenoid
Control		**	**	*	
Exponential phase	30.8 \pm 2.9	23.3 \pm 2.0 ^b	18.5 \pm 1.5 ^b	0.65 \pm 0.04 ^a	0.25 \pm 0.03
Stationary phase	26.1 \pm 1.7	29.3 \pm 2.5 ^a	26.3 \pm 1.4 ^a	0.53 \pm 0.03 ^b	0.32 \pm 0.04
N-	**	**	***	**	**
Exponential phase	29.1 \pm 1.9 ^a	24.1 \pm 1.7 ^b	17.8 \pm 1.5 ^b	0.64 \pm 0.02 ^a	0.24 \pm 0.03 ^b
Stationary phase	17.9 \pm 1.0 ^b	29.0 \pm 2.9 ^a	33.7 \pm 2.8 ^a	0.46 \pm 0.04 ^b	0.33 \pm 0.02 ^a

Mean values significantly different: * $P < 0.05$, a > b; ** $P < 0.01$, a > b; *** $P < 0.001$, a > b. The absence of letters indicates that mean values are not significantly different.

Thus, the start of the stationary growth phase, some two or three days later, probably was promoted by the insufficient availability of nitrogen to the microalgae (Eriksen *et al.*, 2007). A second factor that might have contributed to the start of the stationary growth phase was light availability. The excess of cells possibly produced a self-shading in the cultures and a presumed effect of photoacclimation by the microalgae (Johnsen & Sakshaug, 2007).

Despite the low concentration of nitrogen in the culture medium after seven days of growth, cells continued to divide in relatively high rates (growth rates fluctuated between 0.30 and 0.15) in the transition between the exponential and the stationary growth phases. This may be due the use of internal pools of inorganic nitrogen, which sometimes achieve high concentrations (Lavín & Lourenço, 2005). The capability of accumulating inorganic nitrogen is a key factor to keep phytoplankton alive under fluctuations in nitrogen supply in natural environments (Dortch, 1982). In cultures, microalgae maintain the trend of taking up high concentrations of dissolved inorganic nitrogen supplied by the culture medium. The accumulation of high concentrations of inorganic pools of nitrogen (such as nitrate, nitrite and ammonia/ammonium) in the exponential phase reflects the rapid nitrogen uptake in the first days of growth, when no factor is limiting (Lavín & Lourenço, 2005). Thus, the consumption of intracellular pools of inorganic nitrogen can promote growth of microalgae even under scarce concentrations of nitrogen in the culture medium. This could also explain the lack of differences in final cell yield when the control and the N- treatment of both species are compared.

The assimilatory process of nitrogen may be limited by the activity of enzymes, and the accumulation of high concentrations of intracellular inorganic nitrogen may also be a consequence of this condition. Marine

microalgae may accumulate large transitory pools of inorganic nitrogen when the rates of uptake are higher than growth rates. The accumulation of pools of inorganic nitrogen, such as NO₃⁻, may be a consequence of differences in the rates of the previous step in the assimilatory process (Berges, 1997). Imbalance between up take and assimilation of nitrogen was detected in our experiments. High concentrations of nitrite might have been built up, and excretion of this ion may have occurred preventing its toxic effects, as demonstrated by Lourenço *et al.* (1997) in nitrogen-rich cultures of the prasinophycean *Tetraselmis gracilis*. Lomas & Glibert (2000) also reported the release of nitrite in nitrogen-sufficient cultures of some diatoms and flagellates as a response to rapid increases in irradiance. Concentrations of nitrite in the culture medium in experiments with *Chlorella* sp. increased from almost zero to ~8.0-9.0 μ M after four days of exponential growth, and to ~30.0-40.0 μ M in cultures of *N. oculata*. A similar trend was found by Aidar *et al.* (1991) in experiments run with the diatom *Phaeodactylum tricornutum*. This behaviour results from the reduction of nitrate to nitrite by nitrate reductase activity, not followed by further reduction to ammonia by nitrite reductase (Berges, 1997).

Phosphorus has not limited the growth of the microalgae in the present study. The phosphate concentrations declined throughout all experiments, but in none of them it was depleted. Phosphorus is an integral part of nucleic acids and biological membranes, and it acts as a carrier substrate of chemical energy (ATP) in the cytoplasm (La Roche *et al.*, 1993). The excess of phosphorus in N- experiments could prevent any constraint by energy, considering that ATP-dependent physiological processes would not be limited by phosphorus. In addition, the lack of nitrogen would stimulate the cells to synthesize more carbohydrate and/or lipid (*e.g.*, Rodolfi *et al.*, 2009; Li

et al., 2011). However, our data suggest that even in the control the microalgae had sufficient phosphorus to synthesize all cell constituents and run ATP-dependent physiological processes. Dean *et al.* (2008) demonstrated that cultures of the chlorophycean *Chlamydomonas reinhardtii* run with high concentrations of phosphorus promoted increments in intracellular P quota, as well as more accumulation of both carbohydrate and lipid. Other studies claim that the synthesis of storage products is favoured under phosphorus limitation. Studying the trebouxiophycean *Chlorella vulgaris*, Chia *et al.* (2013) concluded that the accumulation of carbohydrate and lipid was greater under phosphorus limitation. Similarly, Sigee *et al.* (2007) detected more production of carbohydrate and lipid in P-limited cultures of the chlorophycean *Scenedesmus subspicatus*. Our experimental design does not allow us to evaluate if the extremely high availability of phosphorus in the N- experiments had any stimulatory effect on the production of particular substances, such as carbohydrate and lipid.

The dynamics of growth in algal and cyanobacterial cultures is also influenced by changes in cell biovolumes. According to Borges-Campos *et al.* (2010) microalgal species with small cell volumes tend to have higher growth rates in comparison to large-sized cells. This interpretation seems to be coherent here, explaining well the higher growth rate of *N. oculata*, a small-sized species, in comparison to *Chlorella* sp. On the other hand, cell biovolumes did not vary in the control throughout growth, but increments in biovolumes were found only in N- experiments for both species. This trend seems to be related to the progressive reduction of nitrogen concentrations in the culture medium and the ability to accumulate energy reserves (carbohydrate and/or lipid) by the test-species. The accumulation of high concentrations of carbohydrate and/or lipid may contribute to enlarge cells (Dean *et al.*, 2010; Traller & Hildebrand, 2013), since reserves accumulate in the cytoplasm in most of the algal lineages (Graham *et al.*, 2009). Our results indicate that *Chlorella* sp. showed at the 13th day of cultivation cell biovolumes ca. 32% larger in N- experiments than in the control. For *N. oculata*, cell biovolumes in the 13th day of cultivation were ca. 25% larger in N- experiments than in the control. The accumulation of carbohydrate and lipid was remarkable for *Chlorella* sp. and *N. oculata*, respectively, throughout time in N- experiments (see discussion on gross chemical composition of the microalgae). In addition, in the stationary growth phase the lower growth rates reflect in slower cell divisions, which contribute to increase of frequency of more cells with larger cell volumes (Carfagna *et al.*, 2015).

Gross chemical composition

Protein content of the two microalgae exhibited different trends in the experiments. The lack of variation of protein content over growth in the control indicates that the exhaustion of nitrogen in the culture medium, recorded from the 10th day of growth, had no detectable effect on the protein content of the microalgae. A possible explanation for this trend results from the consumption of internal pools of inorganic nitrogen, presumably created due the luxuriant uptake of nitrate (Lourenço *et al.*, 1998). Large pools of nitrate, nitrite and ammonia/ammonium are accumulated in cell vacuoles during the exponential growth of batch cultures run with sufficiency in nitrate (Lomas & Glibert 2000; Lavín & Lourenço, 2005). The consumption of the inorganic pools of nitrogen occurs when dissolved nitrogen declines in the culture medium, and this makes both growth and production of protein viable even with scarce concentrations of nitrogen in the culture medium. This scenario suggests an “apparent” starvation in the control, based on measurements of nitrogenaceous nutrients in the culture medium. The actual nitrogen starvation would be reached only when the internal pools of nitrogen were consumed by the microalgae, normally later in the stationary growth phase. On the other hand, a significant decrease in protein concentrations were recorded from the exponential to the stationary growth phase of N- experiments for both species. This trend suggests that the addition of nutrient solutions (without nitrogen) of the Conway culture medium created an imbalance that affected the production of protein. The high availability of phosphorus, trace metals and other components might have stimulated the synthesis of non-nitrogenaceous substances, such as ATP and carotenoids. The low availability of nitrogen in the stationary growth phase of N- experiments lead to a low protein content, which is in accordance with many other studies (*e.g.*, Young & Beardall, 2003; Setta *et al.*, 2014). Our data on total nitrogen corroborate these interpretations, considering that most of the intracellular nitrogen of marine microalgae is contained in protein (Lourenço *et al.*, 2004). In N- experiments total N was significantly lower than in the control in the stationary growth phase for both species, which points to a physiological stress caused by nitrogen starvation (Young & Beardall, 2003).

Our results for protein content (>30% d.m.) in the control (and the exponential growth phase of the N- experiments) were slightly higher than the values reported by Renaud *et al.* (1999) for 18 microalgae cultured in Australia, which typically ranged from 24 to 29% d.m. This may be a result of different culture conditions and also the efficient protocol of protein

extraction used by us, previously tested with different macro- and microalgae (Barbarino & Lourenço, 2005). Our results for protein content are similar to those presented by Brown (1991) for some species of green algae and *Nannochloropsis oculata*, one of the species studied by us. Our results of protein content in the stationary growth phase are typically lower than those recorded in the literature for microalgae cultured in sufficiency of nutrients (e.g., Lourenço *et al.*, 2002; Natrah *et al.*, 2007; González-López *et al.*, 2010), but they were similar to data of studies run with nitrogen starvation (e.g., Pelah *et al.*, 2004; Silva *et al.*, 2009; Tunçay *et al.*, 2013).

The accumulation of carbohydrate reserves occurred in both species in all experiments in the stationary growth phase. In all comparisons throughout time, concentrations of carbohydrate were higher in older cultures. However, the accumulation of carbohydrate seemed to be more intense in *Chlorella* sp., in comparison to *N. oculata*. For *Chlorella* sp., the concentrations of carbohydrate roughly doubled from the exponential to the stationary growth phases in the control, and a ~2.5-fold increase occurred in N-experiment for the same microalga (Table 2). The increment of carbohydrate in *N. oculata* throughout growth was smaller than in *Chlorella* sp., achieving ca. 31% in the two experiments. These differences are related to taxonomic traits. Green algae have carbohydrate as the main product to store energy, especially as starch. Eustigmatophycean algae also accumulate carbohydrate as reserves, but they produce great amounts of lipid too (Graham *et al.*, 2009). This trend is verified in members of other classes of the algal lineage Heterokontophyta (= Ochrophyta), such as the chrysophyceans, the bolidophyceans, and the pinguiphyceans, among others (Reviere, 2006). Reinforcing the interpretations on the energy reserves produced by different algal lineages, there was no variation in the total lipid produced by the trebouxiophycean *Chlorella* sp. in the two experiments. Nevertheless, the eustigmatophycean *N. oculata* exhibited a ~42% increase in lipid content over growth in the control, and a ~2.1-fold increase in lipid content over growth in the N-experiment (Table 3).

Concentrations of carbohydrate in the exponential growth phase of all experiments were similar to the results published by Lourenço *et al.* (2002) for *Chlorella minutissima* and *N. oculata*, as well as Machado & Lourenço (2008) for green microalgae. In the stationary growth phase of both control and N-experiments our results (>31% d.m. for *N. oculata*; >40% d.m. for *Chlorella* sp.) are higher than most of the available data in the literature (e.g., Brown, 1991; Renaud *et al.*, 1999), but they were similar to results

found in studies run with nitrogen starvation (e.g., Silva *et al.*, 2009; Ikarán *et al.*, 2015).

Concentrations of lipid in *Chlorella* sp. were low, always lower than 15.5% d.m., even in the N-experiment. These results are similar to those reported by different authors for green algae (e.g., Machado & Lourenço, 2008; Li *et al.*, 2008). However, this species did not respond to nitrogen starvation with increases in total lipid. In many other studies, authors were able to detect significant increments of the lipid content of green microalgae under nitrogen starvation (e.g., Tang *et al.*, 2011; Karemore *et al.*, 2013; Urreta *et al.*, 2014). As *Chlorella* sp. failed to produce more lipid under nitrogen starvation, this strain may be unsuitable in applications that need high concentrations of lipid, such as biodiesel production. On the other hand, the production of lipid by *N. oculata* was influenced by the nitrogen availability such as other eukaryotic microalgae (Chen *et al.*, 2011; Suali & Sarbatly, 2012). Particularly in the N-experiment, *N. oculata* almost doubled its lipid content over growth, in a similar trend reported by other studies run with nitrogen starvation (e.g., Rodolfi *et al.*, 2009; Jiang *et al.*, 2011; Kaye *et al.*, 2015). Our results confirm *N. oculata* as a suitable species to be used in applications that need high concentrations of lipid, such as nutrition of some marine animals (Costard *et al.*, 2012).

In general, increments in storage substances throughout growth of both species seems to be related to the progressively lower availability of N in the culture medium. This general trend is widely documented in the literature (e.g., Geider & La Roche, 2002; Silva *et al.*, 2009; Praveenkumar *et al.*, 2012). Production of protein is favoured during periods of nitrogen sufficiency, with limited carbohydrate and lipid synthesis; conversely, during periods of reduced nitrogen availability, carbohydrate and/or lipid accumulate and protein production drops (Guo *et al.*, 2013). On the other hand, the increase of cell volumes over growth probably results mainly of the intense accumulation of non-nitrogenaceous substances, such as carbohydrate and lipid, as discussed in the previous section.

Concentrations of chlorophyll dropped from the exponential to the stationary growth phase of both microalgae in all experiments in a similar way. In the control, the concentrations of chlorophyll decreased ca. 20.0% over growth of *Chlorella* sp. and ca. 18.5% in *N. oculata*. In N-experiments, the concentrations of chlorophyll dropped ca. 28.5% over growth of *Chlorella* sp. and ca. 28.0% in *N. oculata*. These trends are also connected to the availability of nitrogen. Chlorophyll-*a* is a nitrogenaceous substance, with 6.28% N in its molecular mass (Lourenço *et al.*, 1998). Growth under nitrogen limitation generates variations

of chlorophyll content similar to those described for protein: decrease of the percentages throughout time (Bellefeuille *et al.*, 2014). Absolute percentages of chlorophyll-*a* were bigger in *Chlorella* sp. (0.72-1.05% d.w.) than in *N. oculata* (0.46-0.65% d.w.), which is in accordance with taxonomic traits. Chlorophytes are probably the richest algal group in chlorophyll-*a*, which may achieve more than 1.5% d.w. in some species (Machado & Lourenço, 2008). Most of the algal groups typically show less than 0.7% d.w. of chlorophyll-*a* when grown in nutrient sufficiency and even lower concentrations under nitrogen starvation (Lourenço *et al.*, 2004).

Both microalgae showed similar trends of variation of total carotenoid in the experiments. The lack of variation in the control is regarded as a consequence of sufficiency in nutrients to synthesize carotenoids. These substances do not contain nitrogen and their synthesis is not inhibited by the depletion of nitrogen, such as chlorophyll. As the cultures experienced nitrogen limitation for only two or three days in the control (see discussion of the previous section), this probably did not reflect in significant increments in total carotenoid. On the other hand, in N- experiments there was an increase in total carotenoid over growth. This trend is supported by studies run with the chlorophytes *Dunaliella salina* (Lamers *et al.*, 2012) and *Chlorella zofingiensis* (Mulders *et al.*, 2014) and with the haptophyte *Isochrysis galbana* (Roopnarain *et al.*, 2014), among many others. Increments in total carotenoid are possibly a consequence of the need of more photosynthesis antennae to support growth of the species. Chlorophyll-*a* is the main photosynthetic pigment, but under nitrogen starvation it is not possible to produce additional chlorophyll molecules for new daughter-cells. Microalgae can compensate the decrease in chlorophyll producing more carotenoids (Young & Beardall, 2003).

Both microalgae tested here showed a significant increment of total carotenoid over growth in N-experiments. Despite this trend points for potential uses of these microalgae to produce carotenoids, it is necessary to identify their individual carotenoids in order to evaluate if they produce any substance of high value. Commercial applications of carotenoids currently known involve some specific valuable substances, such as astaxanthin, β -carotene, and zeaxanthin, for instance (Spolaore *et al.*, 2006).

Mariculture uses and potential for biofuel production

Our study provided data on growth, nutrient uptake, and gross chemical composition of two microalgae in two experimental conditions. Current results confirm that the two microalgae tested present variable

chemical composition and their gross chemical profile can be changed depending on the experimental conditions. We used here a two-step cultivation, creating a physiological condition of nitrogen starvation after the cultures achieve high cell densities. This strategy combines the need of high biomass and the induction of the synthesis of substances of interest. A growth under nitrogen limitation since the beginning of the cultures would not provide enough biomass for possible applications.

The high concentrations of lipid showed by *N. oculata* are suitable for the use of this microalga as a food-species in mariculture and it is also favorable for the potential production of biodiesel. Cultivation under nitrogen starvation provided significant increments in total lipid, which is important to feed animals with a high demand for fatty food, such as larvae of oysters (Martínez-Fernández *et al.*, 2006). Under nitrogen starvation, *N. oculata* exhibited low concentrations of protein, which may be unsuitable for some animal species. In theory, this possible deficiency in protein can be compensated if *N. oculata* is offered in diets combined with a protein-rich microalga of similar size and shape. On the other hand, a possible future use of *N. oculata* as a candidate feedstock to produce biodiesel is strengthened by the current results, considering that its chemical composition could be successfully changed to produce more lipid.

The new strain tested here, *Chlorella* sp., exhibited a good productivity of biomass, but its low concentration of lipid may limit its use both to feed some marine animals and as well as to convert its biomass in biodiesel. The high concentration of protein presented by *Chlorella* sp. may be suitable to feed marine animals with high demand for protein (Brown *et al.*, 1997). Trials to assess the digestibility of its cell wall are needed to better evaluate its use in mariculture. Currently there are many research groups creating new photobioreactors that can be more efficient to produce microalgae biomass and even specific substances, such as lipids (Rodolfi *et al.*, 2009). However, even with the expected progress to produce microalgae biomass it is unlike that low-lipid strains can be selected as feedstocks for biodiesel production, considering the tremendous technological challenges of this enterprise (Davis *et al.*, 2011). The high content of carbohydrate of *Chlorella* sp. may be potentially useful to produce bioethanol, which technical and economic viabilities still have to be demonstrated.

In the next step of our research, the fatty acid profile of the microalgae tested here will be evaluated in different experimental conditions, which will be important to better interpret their possible uses.

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REFERENCES

- Aidar, E., R. Ehrlich, C.S. Asano & T.C.S. Sigaud. 1991. Variação da composição bioquímica de *Phaeodactylum tricornutum* (Bohlin), em cultivos estanques. Bolm. Inst. Oceanogr. São Paulo, 39(2): 131-139.
- Aminot, A. & M. Chaussepied. 1983. Manuel des analyses chimiques en milieu marin. CNEXO, Brest, 395 pp.
- Barbarino, E. & S.O. Lourenço. 2005. An evaluation of methods for extraction and quantification of protein from marine macro- and microalgae. J. Appl. Phycol., 17: 447-460.
- Bellefeuille, S.D., S. Dorion, J. Rivoal & D. Morse. 2014. The dinoflagellate *Lingulodinium polyedrum* responds to N depletion by a polarized deposition of starch and lipid bodies. PLoS ONE, 9(11): e111067.
- Berges, J.A. 1997. Algal nitrate reductases. Eur. J. Phycol. 32(1): 3-8.
- Borges-Campos, V., E. Barbarino & S.O. Lourenço. 2010. Crescimento e composição química de dez espécies de microalgas marinhas em cultivos estanques. Cienc. Rural, 40(2): 339-347.
- Brown, M.R. 1991. The amino-acid and sugar composition of 16 species of microalgae used in mariculture. J. Exp. Mar. Biol. Ecol., 145: 79-99.
- Brown, M.R., S.W. Jeffrey, J.K. Volkman & G.A. Dunstan. 1997. Nutritional properties of microalgae for mariculture. Aquaculture, 151(1-4): 315-331.
- Carfagna, S., G. Salbitani, C. Bottone, A. De Marco & V. Vona. 2015. Cross-effects of nitrogen and sulphur starvation in *Chlorella sorokiniana* 211/8K. Nat. Resour., 6: 221-229.
- Carvalho, A.P., C.M. Monteiro & F.X. Malcata. 2009. Simultaneous effect of irradiance and temperature on biochemical composition of the microalga *Pavlova lutheri*. J. Appl. Phycol., 21(5): 543-552.
- Chen, M., H. Tang, H. Ma, T.C. Holland, K.Y.S. Ng & S.T. Salley. 2011. Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*. Bioresource Technol., 102: 1629-1655.
- Chia, M.A., A.T. Lombardi & M.G.G. Melão. 2013. Calorific values of *Chlorella vulgaris* (Trebouxiophyceae) as a function of different phosphorus concentrations. Phycological Res., 61(4): 286-291.
- Chiu, S.-Y., C.-Y. Kao, M.-T. Tsai, S.-C. Ong, C.-H. Chen & C.-S. Lin. 2009. Lipid accumulation and CO₂ utilization of *Nannochloropsis oculata* in response to CO₂ aeration. Bioresource Technol., 100: 833-838.
- Costard, G.S., R.R. Machado, E. Barbarino, R.C. Martino & S.O. Lourenço. 2012. Chemical composition of five marine microalgae that occur on the Brazilian coast. Int. J. Fish. Aquacult., 4(9): 191-201.
- Davis, R., A. Aden & P.T. Pienkos. 2011. Techno-economic analysis of autotrophic microalgae for fuel production. Appl. Energy, 88(10): 3524-3531.
- Dean, A.P., J.M. Nicholson & D.C. Sigeo. 2008. Impact of phosphorus quota and growth phase on carbon allocation in *Chlamydomonas reinhardtii*: an FTIR microspectroscopy study. Eur. J. Phycol., 43(4): 345-354.
- Dean, A.P., D.C. Sigeo, B. Estrada & J.K. Pittman. 2010. Using FTIR spectroscopy for rapid determination of lipid accumulation in response to nitrogen limitation in freshwater microalgae. Bioresource Technol., 101: 4499-4507.
- Dortch, Q. 1982. Effect of growth conditions on accumulation of internal nitrate, ammonium, amino acids, and protein in three marine diatoms. J. Exp. Mar. Biol. Ecol., 61: 243-264.
- Doucha, J., F. Straka & K. Lívanský. 2005. Utilization of flue gas for cultivation of microalgae (*Chlorella* sp.) in an outdoor open thin-layer photobioreactor. J. Appl. Phycol., 17(5): 403-412.
- Dubois, M., K.A. Gilles, J.K. Hamilton, P.A. Rebers & F. Smith. 1956. Colorimetric method for the determination of sugars and related substances. Anal. Chem., 18: 350-356.
- Durmaz, Y., M. Donato, M. Monteiro, L. Gouveia, M.L. Nunes, T. Gama Pereira, S. Gökpınar, N.M. Bandarra. 2009. Effect of temperature on α -tocopherol, fatty acid profile, and pigments of *Diacronema vlkianum* (Haptophyceae). Aquacult. Int. 17: 391-399.
- Eriksen, N.T., B.R. Poulsen & J.J. Lønsmann Iversen. 1998. Dual sparging laboratory-scale photobioreactor for continuous production of microalgae. J. Appl. Phycol., 10(4): 377-382.
- Eriksen, N.T., F. Riisgård, W.S. Gunther & J.J.L. Iversen. 2007. On-line estimation of O₂ production, CO₂ uptake, and growth kinetics of microalgal cultures in a gas-tight photobioreactor. J. Appl. Phycol., 19(2): 161-174.
- Falkowski, P.G. & J.A. Raven. 2007. Aquatic photosynthesis. Princeton University Press, Princeton, 484 pp.

- Faria, G.R., C.R.P.S. Paes, D.J.F.A. Castro, N.A.B. Tinoco, E. Barbarino & S.O. Lourenço. 2012. Effects of the availability of CO₂ on growth, nutrient uptake, and chemical composition of the marine microalgae *Chlorella* sp. and *Nannochloropsis oculata*, two potentially useful strains for biofuel production. *Int. Res. J. Biotechnol.*, 3(5): 65-75.
- Fidalgo, J.P., A. Cid, J. Abalde & C. Herrero. 1995. Culture of the marine diatom *Phaeodactylum tricorutum* with different nitrogen sources: growth, nutrient conversion and biochemical composition. *Cah. Biol. Mar.*, 36: 165-173.
- Folch, J., M. Lees & G.M. Sloane-Stanley. 1957. A simple method for the isolation and purification of total lipid from animal tissues. *J. Biol. Chem.*, 226: 497-509.
- Geider, R., J. La Roche, R. Greene & M. Olaizola. 1993. Response of the photosynthetic apparatus of *Phaeodactylum tricorutum* (Bacillariophyceae) to nitrate, phosphate, or iron starvation. *J. Phycol.* 29: 755-66.
- Geider, R. & J. La Roche. 2002. Redfield revisited: variability of C:N:P in marine microalgae and its biochemical basis. *Eur. J. Phycol.*, 37(1): 1-17.
- Goldman, J.C., Y. Azov, C.B. Riley & M.R. Dennett. 1982. The effect of pH in intensive microalgal cultures. I. Biomass regulation. *J. Exp. Mar. Biol. Ecol.*, 57: 1-13.
- González López, C.V., M.C. Cerón García, F.G. Acién Fernández, C. Segovia Bustos, Y. Chisti & J.M. Fernández-Sevilla. 2010. Protein measurements of microalgal and cyanobacterial biomass. *Bioresource Technol.*, 101(19): 7587-7591.
- Graham, L.E., J.E. Graham & L.W. Wilcox. 2009. *Algae*. Benjamin Cummings (Pearson), San Francisco, 720 pp.
- Grasshoff, K., M. Ehrhardt & K. Kremling. 1983. *Methods of seawater analysis*. Verlag Chemie, Weinheim, 419 pp.
- Grobbelaar, J.U. 2014. Microalgal biomass production: challenges and realities. *Photosynth. Res.*, 106(1-2): 135-144.
- Guillard, R.R.L. 1975. Culture of phytoplankton for feeding marine invertebrates. In: W.L. Smith & M.H. Chanley (eds.). *Culture of marine invertebrate animals*. Plenum Publishing Corporation, New York, pp. 29-60.
- Guo, H., M. Daroch, L. Liu, G. Qiu, S. Geng & G. Wang. 2013. Biochemical features and bioethanol production of microalgae from coastal waters of Pearl River Delta. *Bioresource Technol.*, 127: 422-428.
- Hach, C.C., B.K. Bowden, A.B. Kopelove & S.V. Brayton. 1987. More powerful peroxide Kjeldahl digestion method. *J. Assoc. Off. Anal. Chem.*, 70(5): 783-787.
- Harrison, P.J. & J.A. Berges. 2005. *Marine culture media*. In: R.A. Andersen (ed.). *Algal culturing techniques*. Academic Press, San Diego, pp. 21-33.
- Hillebrand, H., C.-D. Dürselen, D. Kirschtel, U. Pollinger & T. Zohary. 1999. Biovolume calculation for pelagic and benthic microalgae. *J. Phycol.*, 35(2): 403-424.
- Huerlimann, R., R. de Nys & K. Heimann. 2010. Growth, lipid content, productivity, and fatty acid composition of tropical microalgae for scale-up production. *Biotechnol. Bioeng.*, 107(2): 245-257.
- Ikan, Z., S. Suárez-Alvarez, I. Urreta & S. Castañón. 2015. The effect of nitrogen limitation on the physiology and metabolism of *Chlorella vulgaris* var L3. *Algal Res.*, 10: 134-144.
- Jeffrey, S.W. & G.F. Humphrey. 1975. New spectrophotometric equations for determining chlorophylls a, b, c1 and c2 in higher plants, algae and natural phytoplankton. *Biochem. Physiol. Pflanz.*, 167: 191-194.
- Jiang, L., S. Luo, X. Fan, Z. Yang & R. Guo. 2011. Biomass and lipid production of marine microalgae using municipal wastewater and high concentration of CO₂. *Appl. Energ.*, 88: 3336-3341.
- Johnsen, G. & E. Sakshaug. 2007. Biooptical characteristics of PSII and PSI in 33 species (13 pigment groups) of marine phytoplankton, and the relevance for pulse-amplitude-modulated and fast-repetition-rate fluorometry. *J. Phycol.*, 43(6): 1236-1251.
- Karemore, A., R. Pal & R. Sen. 2013. Strategic enhancement of algal biomass and lipid in *Chlorococcum infusionum* as bioenergy feedstock. *Algal Res.*, 2(2): 113-121.
- Kaye, Y., O. Grundman, S. Leu, A. Zarka, B. Zorin, S. Didi-Cohen, I. Khozin-Goldberg & S. Boussiba. 2015. Metabolic engineering toward enhanced LC-PUFA biosynthesis in *Nannochloropsis oceanica*: Overexpression of endogenous $\Delta 12$ desaturase driven by stress-inducible promoter leads to enhanced deposition of polyunsaturated fatty acids in TAG. *Algal Res.*, 11: 387-398.
- Kester, D.R., I.W. Duedall, D.N. Connors & R.M. Pytkowicz. 1967. Preparation of artificial seawater. *Limnol. Oceanogr.*, 12(1): 176-179.
- Lamers, P.P., M. Janssen, R.C.H. De Vos, R.J. Bino & R.H. Wijffels. 2012. Carotenoid and fatty acid metabolism in nitrogen-starved *Dunaliella salina*, a unicellular green microalga. *J. Biotechnol.*, 162(1): 21-27.
- La Roche, J., R.J. Geider, L.M. Graziano, H. Murray & K. Lewis. 1993. Induction of specific proteins in eukaryotic algae grown under iron-, phosphorus-, or nitrogen-deficient conditions. *J. Phycol.*, 29: 767-777.
- Lavín, P.L. & S.O. Lourenço. 2005. An evaluation of the accumulation of intracellular inorganic nitrogen pools

- by marine microalgae in batch cultures. *Braz. J. Oceanogr.*, 53(1/2): 55-68.
- Lee-Chang, K.J., P.D. Nichols & S.I. Blackburn. 2013. More than biofuels-potential uses of microalgae as sources of high-value lipids. *Lipid Technol.*, 25(9): 199-203.
- Li, Y., M. Horsman, B. Wang, N. Wu & C. Lan. 2008. Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochloris oleoabundans*. *Appl. Microbiol. Biotechnol.*, 81(4): 629-636.
- Li, Z.S., H.L. Yuan, J.S. Yang & B.Z. Li. 2011. Optimization of the biomass production of oil algae *Chlorella minutissima* UTEX2341. *Bioresource Technol.*, 102(19): 9128-9134.
- Lim, D.K.Y., S. Garg, M. Timmins, E.S.B. Zhang, S.R. Thomas-Hall, H. Schuhmann, Y. Li & P.M. Schenk. 2012. Isolation and evaluation of oil-producing microalgae from subtropical coastal and brackish waters. *PLoS ONE*, 7(7): e40751.
- Lomas, M.W. & P.M. Glibert. 2000. Comparisons of nitrate uptake, storage, and reduction in marine diatoms and dinoflagellates. *J. Phycol.*, 36: 903-913.
- Lourenço, S.O. & A.A.H. Vieira. 2004. Culture collections of microalgae in Brazil: progress and constraints. *Nova Hedwigia*, 79(1-2): 149-173.
- Lourenço, S.O., E. Barbarino, U.M. Lanfer-Marquez & E. Aidar. 1998. Distribution of intracellular nitrogen in marine microalgae: basis for the calculation of specific nitrogen-to-protein conversion factors. *J. Phycol.*, 34(5): 798-811.
- Lourenço, S.O., E. Barbarino, A. Nascimento & R. Paranhos. 2005. Seasonal variations in tissue nitrogen and phosphorus of eight macroalgae from a tropical hypersaline coastal environment. *Cryptogamie*, 26(4): 355-371.
- Lourenço, S.O., U.M. Lanfer-Marquez, J. Mancini-Filho, E. Barbarino & E. Aidar. 1997. Changes in biochemical profile of *Tetraselmis gracilis*. I. Comparison of two culture media. *Aquaculture*, 148(2/3): 153-168.
- Lourenço, S.O., E. Barbarino, J. Mancini-Filho, K.P. Schinke & E. Aidar. 2002. Effects of different nitrogen sources on growth and biochemical profile of ten marine microalgae under batch cultures: an evaluation for aquaculture. *Phycologia*, 41(2): 158-168.
- Lourenço, S.O., E. Barbarino, P.L. Lavín, U.M.L. Marquez & E. Aidar. 2004. Distribution of intracellular nitrogen in marine microalgae. Calculation of new nitrogen-to-protein conversion factors. *Eur. J. Phycol.*, 39(1): 17-32.
- Lourenço, S.O., E. Barbarino, M.G. Bispo, D.A. Borges, C. Coelho-Gomes, P.L. Lavín & F. Santos. 2008. Effects of light intensity on growth, inorganic nitrogen storage, and gross chemical composition of four marine microalgae in batch cultures. *Museu Nacional, Série Livros*, 30: 203-214.
- Lowry, O.H., N.J. Rosebrough, A.L. Farr & R.L. Randall. 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem.*, 193: 265-275.
- Machado, R.R. & S.O. Lourenço. 2008. Propriedades nutricionais de microalgas usadas como alimento de moluscos bivalves: uma revisão. *Museu Nacional, Série Livros*, 30: 281-304.
- MacIntyre, H.L. & J.J. Cullen. 2005. Using cultures to investigate the physiological ecology of microalgae. In: R.A. Andersen (ed.). *Algal culturing techniques*. Academic Press, San Diego, pp. 287-326.
- Maity, J.P., J. Bundschuh, C.-Y. Chen & P. Bhattacharya. 2014. Microalgae for third generation biofuel production, mitigation of greenhouse gas emissions and wastewater treatment: present and future perspectives - a mini review. *Energy*, 78: 104-113.
- Martínez-Fernández, E., H. Acosta-Salmón & P.C. Southgate. 2006. The nutritional value of seven species of tropical microalgae for black-lip pearl oyster (*Pinctada margaritifera*, L.) larvae. *Aquaculture*, 257(1-4): 491-503.
- Myklestad, S. & A. Haug. 1972. Production of carbohydrates by the marine diatom *Chaetoceros affinis* var. *willei* (Gran) Hustedt. I. Effect of the concentration of nutrients in the culture medium. *J. Exp. Mar. Biol. Ecol.*, 9: 125-136.
- Mubarak, M., A. Shaija & T.V. Suchithra. 2015. A review on the extraction of lipid from microalgae for biodiesel production. *Algal Res.*, 7: 117-123.
- Mulders, K.J.M., J.H. Janssen, D.E. Martens, R.H. Wijffels & P.P. Lamers. 2014. Effect of biomass concentration on secondary carotenoids and triacylglycerol (TAG) accumulation in nitrogen-depleted *Chlorella zofingiensis*. *Algal Res.*, 6: 8-16.
- Natrah, F.M.I., F.M. Yusoff, M. Shariff, F. Abas & N.S. Mariana. 2007. Screening of Malaysian indigenous microalgae for antioxidant properties and nutritional value. *J. Appl. Phycol.*, 19(6): 711-718.
- Pelah, D., I. Marton, W. Wang, O. Shoseyov, A. Altman & E. Cohen. 2004. Accumulation and protection activity of protease-resistant heat-stable proteins in *Haematococcus pluvialis* during high light and nitrogen starvation. *J. Appl. Phycol.*, 16(2): 153-156.
- Praveenkumar, R., K. Shameera, G. Mahalakshmi, M.A. Akbarsha & N. Thajuddin. 2012. Influence of nutrient deprivations on lipid accumulation in a dominant indigenous microalga *Chlorella* sp., BUM11008: evaluation for biodiesel production. *Biomass Bioenergy*, 37: 60-66.
- Renaud, S.M., L.-V. Thinh & D.L. Parry. 1999. The gross chemical composition and fatty acid composition of 18

- species of tropical Australian microalgae for possible use in mariculture. *Aquaculture*, 170(2): 147-159.
- Ratti, S., A.H. Knoll & M. Giordano. 2013. Grazers and phytoplankton growth in the oceans: an experimental and evolutionary perspective. *PLoS ONE*, 8(11): e77349.
- Reviere, B. 2006. *Biologia e filogenia das algas*. Artmed, Porto Alegre, 280 pp.
- Rodolfi, L., G.C. Zitelli, N. Bassi, G. Padovani, N. Biondi, G. Bonini & M.R. Tredici. 2009. Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. *Biotechnol. Bioeng.*, 102: 100-112.
- Roopnarain, A., V.M. Gray & S. Sym. 2014. Influence of nitrogen stress on *Isochrysis galbana* strain U4, a candidate for biodiesel production. *Phycol. Res.*, 62(4): 237-249.
- Sepúlveda, C., F.G. Ación, C. Gómez, N. Jiménez-Ruiz, C. Riquelme & E. Molina-Grima. 2015. Utilization of centrate for the production of the marine microalgae *Nannochloropsis gaditana*. *Algal Res.*, 9: 107-116.
- Setta, B.R.S., E. Barbarino, F.B. Passos & S.O. Lourenço. 2014. An assessment of the usefulness of the cyanobacterium *Synechococcus subsalsus* as a source of biomass for biofuel production. *Lat. Am. J. Aquat. Res.*, 42(2): 364-375.
- Sigeo, D.C., F., Bahrami, B. Estrada, R.E. Webster & A.P. Dean. 2007. The influence of phosphorus availability on carbon allocation and P quota in *Scenedesmus subspicatus*: a synchrotron-based FTIR analysis. *Phycologia*, 46(5): 583-592.
- Silva, A.F., S.O. Lourenço & R.M. Chaloub. 2009. Effects of nitrogen starvation on the photosynthetic physiology of a tropical marine microalga *Rhodomonas* sp. (Cryptophyceae). *Aquacult. Bot.*, 91(4): 291-297.
- Spolaore, P., C. Joannis-Cassan, E. Duran & A. Isambert. 2006. Commercial applications of microalgae. *J. Biosci. Bioeng.*, 101(2): 87-96.
- Strickland, J.D.H. & T.R. Parsons. 1968. A practical handbook of seawater analysis. *Bull. Fish. Res. Bd. Can.*, 167: 1-311.
- Suali, E. & R. Sarbatly. 2012. Conversion of microalgae to biofuel. *Renew. Sust. Energ. Rev.*, 16(6): 4316-4342.
- Sunda, W.G., N.M. Price & F.M.M. Morel. 2005. Trace metal ion buffers and their use in culture studies. In: R.A. Andersen (ed.). *Algal culturing techniques*. Academic Press, San Diego, pp. 35-63.
- Tang, H., M. Chen, M.E.D. Garcia, N. Abunasser, K.Y.S. Ng & S.O. Salley. 2011. Culture of microalgae *Chlorella minutissima* for biodiesel feedstock production. *Biotechnol. Bioeng.*, 108(10): 2280-2287.
- Templeton, D.W. & L.M.L. Laurens. 2015. Nitrogen-to-protein conversion factors revisited for applications of microalgal biomass conversion to food, feed and fuel. *Algal Res.*, 11: 359-367.
- Tokuşoglu, Ö. & M.K. Ünal. 2003. Biomass nutrient profiles of three microalgae: *Spirulina platensis*, *Chlorella vulgaris*, and *Isochrysis galbana*. *J. Food Sci.*, 68(4): 1144-1148.
- Traller, J.C. & M. Hildebrand. 2013. High throughput imaging to the diatom *Cyclotella cryptica* demonstrates substantial cell-to-cell variability in the rate and extent of triacylglycerol accumulation. *Algal Res.*, 2(3): 244-252.
- Tunçay, H., J. Findinier, T. Duchêne, V. Coge, C. Cousin, G. Peltier, S.G. Ball & D. Dauvillée. 2013. A forward genetic approach in *Chlamydomonas reinhardtii* as a strategy for exploring starch catabolism. *PLoS ONE*, 8(9): e74763.
- Turpin, D.H. 1991. Effects of inorganic N availability on algal photosynthesis and carbon metabolism. *J. Phycol.*, 27(1): 14-20.
- Urreta, I., Z. Ikaran, I. Janices, E. Ibañez, M. Castro-Puyana, S. Castañón & S. Suárez-Alvarez. 2014. Revalorization of *Neochloris oleoabundans* biomass as source of biodiesel by concurrent production of lipids and carotenoids. *Algal Res.*, 5: 16-22.
- Valenzuela-Espinoza, E., R. Millán-Núñez & F. Núñez-Cebrero. 1999. Biomass production and nutrient uptake by *Isochrysis* aff. *galbana* (Clone T-ISO) cultured with a low cost alternative to the f/2 medium. *Aquacult. Eng.*, 20: 135-147.
- Van Iersel, S. & A. Flammini. 2010. *Algae-based biofuels: applications and Co-Products*. FAO, Rome, 117 pp.
- Walne, P.R. 1966. Experiments in the large scale culture of the larvae of *Ostrea edulis*. *Fish. Invest. Lond. Ser.*, (2)25: 1-53.
- Wijffels, R.H. & M.J. Barbosa. 2010. An outlook on microalgal biofuels. *Science*, 329: 796-799.
- Wijffels, R.H., O. Kruse & K.J. Hellingwerf. 2013. Potential of industrial biotechnology with cyanobacteria and eukaryotic microalgae. *Curr. Opin. Biotech.*, 24: 405-413.
- Wood, A.M., R.C. Everroad & L.M. Wingard. 2005. Measuring growth rates in microalgal cultures. In: R.A. Andersen (ed.). *Algal culturing techniques*. Academic Press, San Diego, pp. 269-285.
- Young, E.B. & J. Beardall. 2003. Photosynthetic function in *Dunaliella tertiolecta* (Chlorophyta) during a nitrogen starvation and recovery cycle. *J. Phycol.*, 39(5): 897-905.
- Zar, J.H. 1996. *Biostatistical analysis*. Prentice Hall, Englewood Cliffs, 662 pp.

Zeng, X., M.K. Danquah, X.D. Chen & Y. Lu. 2011. Microalgae bioengineering: from CO₂ fixation to biofuel production. *Renew. Sust. Energ. Rev.*, 15(6): 3252-3260.

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