

Research Article

An assessment of the usefulness of the cyanobacterium *Synechococcus subsalsus* as a source of biomass for biofuel production

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ABSTRACT. Nowadays algal biofuels are considered one of the most promising solutions of global energy crisis and climate change for the years to come. By manipulation of the culture conditions, many algal species can be induced to accumulate high concentrations of particular biomolecules and can be directed to the desired output for each fuel. In this context, the present study involved the assessment of the effects of CO₂ availability and nitrogen starvation on growth and chemical composition of the cyanobacterium *Synechococcus subsalsus*, testing a fast-growing native strain. The control experiments were performed with Conway culture medium in 12-day batch cultures, in 6-liter flasks and 12 h photoperiod, with addition of 2 L min⁻¹ filtered air to each flask. Other two experimental conditions were also tested: (i) the placement into the cultures of additional dissolved nutrients except nitrogen, one week after the start of growth (N-), and (ii) the input of pure CO₂ into the flasks from the 5th day of growth (C+). In all cultures, daily cell counts were done throughout the cultivation, as well as measurements of pH and cell biovolumes. Maximum cell yield were found in N-experiments, while cell yields of C+ and control were similar. Dissolved nitrogen was exhausted before the end of the experiments, but dissolved phosphorus was not totally consumed. Protein and chlorophyll-*a* concentrations decreased from the exponential to the stationary growth phase of all experiments, except for protein in the control. In all experiments, carbohydrate, lipid and total carotenoid increased from the exponential to the stationary growth phase, as an effect of nitrogen limitation. Increments in carbohydrate concentrations were remarkable, achieving more than 42% of the dry weight (dw), but concentrations of lipid were always lower than 13% dw. The addition of pure CO₂ did not cause a significant increase in biomass of *S. subsalsus* nor generated more lipid and carbohydrate than the other treatments. Nitrogen starvation caused an intense accumulation of carbohydrate, but the increments of lipid were small. Despite the fast growth, the cyanobacterium *S. subsalsus* has a virtually null potential for biodiesel production, given its low lipid concentrations. The high concentrations of carbohydrate combined with fast growth point to the potential use of this species as raw material for other possible biotechnological processes, after a demonstration of technical and economic viability.

Keywords: biodiesel, biomass, cultivation, lipid, microalgae, cyanobacteria, productivity.

Evaluación de la utilidad de la cianobacteria marina *Synechococcus subsalsus* como fuente de biomasa para la producción de biocombustibles

RESUMEN. Actualmente los biocombustibles elaborados con algas se consideran una de las soluciones más prometedoras de la crisis energética mundial y el cambio climático para los años venideros. Mediante la manipulación de las condiciones de cultivo, muchas especies de algas pueden ser inducidas para acumular altas concentraciones de biomoléculas particulares que se pueden dirigir a la producción de diferentes combustibles. En este contexto, el presente estudio consistió en la evaluación de los efectos de la disponibilidad de CO₂ y falta de nitrógeno sobre el crecimiento y la composición química de la cianobacteria *Synechococcus subsalsus*, probando una cepa nativa de rápido crecimiento. Los experimentos de control se realizaron con medio de cultivo Conway en cultivos durante 12 días, en matraces de 6 L y 12 h de fotoperíodo, con la adición de 2 L min⁻¹ de aire filtrado a cada matraz. También se ensayaron otras dos condiciones experimentales: (i) la colocación en los cultivos de nutrientes disueltos adicionales excepto de nitrógeno, una semana después del inicio del crecimiento (N-), y (ii) la adición de CO₂ puro en los matraces desde el quinto día de crecimiento (C+). En todos los cultivos, los recuentos de células se realizaron a diario durante todo el cultivo, así como mediciones de pH y biovolumen de células. El rendimiento celular máximo se encontró en

los experimentos N-, mientras que los rendimientos celulares de C+ y del control fueron similares. El nitrógeno disuelto se agotó antes del final de los experimentos, pero el fósforo disuelto no se consumió totalmente. Las concentraciones de proteínas y clorofila-*a* disminuyeron de la fase exponencial a la fase estacionaria de crecimiento en todos los experimentos, excepto para la proteína en el control. En todos los experimentos, los hidratos de carbono, lípidos y carotenoides totales se incrementaron de la fase exponencial a la fase de crecimiento estacionario, como un efecto de limitación de nitrógeno. Incrementos en las concentraciones de hidratos de carbono fueron notables, alcanzando más del 42% del peso en seco (ps), pero las concentraciones de lípidos fueron siempre inferiores a 13% peso seco. La adición de CO₂ puro no causó un aumento significativo en la biomasa de *S. subsalsus* ni generó más lípidos e hidratos de carbono que los otros tratamientos. La falta de nitrógeno causó una intensa acumulación de hidratos de carbono, pero los incrementos de lípidos fueron pequeños. A pesar del rápido crecimiento, la cianobacteria *S. subsalsus* tiene un potencial prácticamente nulo para la producción de biodiesel, debido a sus bajas concentraciones de lípidos. Las altas concentraciones de hidratos de carbono combinadas con crecimiento rápido apuntan para el uso potencial de esta especie como materia prima para otros procesos biotecnológicos posibles, después de una demostración de la viabilidad técnica y económica

Palabras clave: biodiesel, biomasa, cultivo, lípidos, microalgas, cianobacteria, productividad.

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INTRODUCTION

Microalgae and cyanobacteria are dominant photosynthetic organisms in the oceans and in most continental aquatic systems, accounting for an enormous contribution to the Earth's global primary productivity (Graham *et al.*, 2009). Through their metabolic activities, they exert a remarkable influence on the biogeochemical cycles, affecting the availability of several chemical elements in nature (Falkowski *et al.*, 1998). As fundamental components of many ecosystems, they also constitute a major source of food for organisms at the base of the food web (Lee Chang *et al.*, 2013). In addition to their key environmental importance, there are some economic uses of microalgae (hereafter including cyanobacteria for simplicity), such as their utilization as food for both animal and human nutrition, the extraction of valuable substances for industrial use (*e.g.*, pigments), and the production of stable isotope biochemicals (Spolaore *et al.*, 2006), for instance.

Microalgae are the most efficient photosynthetic known organisms (Richmond, 2004), with very high growth rates under optimum culture conditions. This suggests a possible increase and diversification of applications involving them in the future, considering the current search for more productive systems to supply bulk products to the society (Pulz & Gross, 2004), in a context of sustainable production using modern biotechnological developments (Wijffels *et al.*, 2013).

The human demand for energy has been increasing continuously due to population growth and industrialization (Lange *et al.*, 2010). Fossil fuels represent *ca.* 70% of the total global energy requirements, parti-

cularly in transportation, manufacturing, and domestic heating (Mata *et al.*, 2010). The continued use of petroleum-based fuels is now widely recognized as unsustainable because of the expected future depletion of the oil fields and the dramatic effects of fossil fuels on the accumulation of carbon dioxide in the environment, leading to both global warming (Afify *et al.*, 2010) and ocean acidification (Doney *et al.*, 2012). Thus, there is a need for alternative renewable sources of fuels, which can represent either a lower carbon footprint or even carbon-neutral products (Wang *et al.*, 2008; Davis *et al.*, 2010).

Sustainable energy production represents one of the most formidable problems of the 21st century, and plant-based biofuels offer a significant promise (Smith *et al.*, 2010). Microalgae-based biofuels, called third-generation biofuels, devoid of the main drawbacks associated with both first and second generation biofuels (Singh *et al.*, 2011), which are produced from seeds, grains, or sugars and derived from lignocellulosic biomass, respectively. In addition, microalgae can be grown using land and water unsuitable for crop plant or food production, unlike some other first and second generation biofuel feedstocks (Clarens *et al.*, 2010). In theory, biofuels from microalgae allow a very high yield per unit area and does not compete with food crops (Rittmann, 2008).

Despite a variety of specific algae-based fuels are technically feasible (*e.g.*, biodiesel, biojetfuel, biogasoline, bioethanol, etc.), this application is not economically viable yet, mainly due to high costs associated with production, harvesting, and extraction of the algal chemical contents (Wijffels & Barbosa, 2010). Several bottlenecks indeed exist at present that preclude the full industrial exploitation of microalgal

cells. Technological improvements of such processes are thus critical, and this will require a multiple approach, including feedstocks, bioreactors, culturing, and better biocatalysts, among others (Amaro *et al.*, 2011; Faria *et al.*, 2012). Considering that the development of microalgae-based biofuels is a huge challenge, it is advisable that research groups keep focus in some key problems of the whole process (Faria *et al.*, 2012).

Biodiesel is a clean-burning fuel produced from grease, vegetable oils, or animal fats. Biodiesel is produced by transesterification of oils with short-chain alcohols or by the esterification of fatty acids (Vasudevan & Briggs, 2008). Microalgae species produce lipids in variable concentrations, however some species can contain high concentrations of it in their dry weight. Microalgae appear to be the only source of renewable biodiesel that is capable of meeting the global demand for transport fuels (Demirbas, 2011), especially if the species combine fast growth and high content of lipids (Lam & Lee, 2012). Experimental studies are needed to optimize both biomass and oil production of microalgae (Li *et al.*, 2011).

The viability of microalgae-based biodiesel industry depends on the selection of suitable strains in regard to profitable yields and oil quality, thus the bioprospection of potentially productive strains is very important (Nascimento *et al.*, 2013). On the other hand, increments in lipid productivity may be attained through proper induction, such as that represented by nitrogen deprivation (Rodolfi *et al.*, 2009; Praveenkumar *et al.*, 2012). Previous studies performed with the cyanobacterium *Synechococcus subsalsus* revealed high growth rates, despite its lipid content is relatively low (Lourenço *et al.*, 2002, 2004). However, the experiments run by Lourenço *et al.* (2002-2004) employed conditions that we assume were not suitable to stimulate the production of lipids. We hypothesized that an improvement in the lipid content of *S. subsalsus* could be achieved if this cyanobacterium were cultured under either high availability of carbon or deprivation of nitrogen.

This study was designed to determine growth, nutrient uptake, and chemical composition of the marine cyanobacterium *Synechococcus subsalsus* under culture conditions that could induce increments in the lipid productivity. We tested a two-phase culture system with either nitrogen starvation or high availability of CO₂ in the second part of the experiments. We discuss if *S. subsalsus* could be a candidate for biodiesel production.

MATERIALS AND METHODS

The cyanobacterium tested

The strain was obtained from Elizabeth Aidar Microalgae Culture Collection, Fluminense Federal University, Brazil (Lourenço & Vieira, 2004). *Synechococcus subsalsus* Skuja, 1939 (Cyanobacteria, strain UB07) was isolated on the northern coast of São Paulo State, Brazil.

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-liter borosilicate flasks. The species was cultured in three experimental conditions:

- a) Seawater enriched with Conway nutrient solution (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 culture conditions described above, including the intermittent daily addition of pure CO₂ from the 5th day of growth, in an average of 10 L day⁻¹. The CO₂ was added manually in each experimental flask, three to five times per day in different moments of the photoperiod (intervals at every 2.0 to 3.5 h). In the first days of addition of pure CO₂, when the cyanobacterium biomass was still small, this procedure was done only three times; the number of additions of CO₂ into the flasks increased throughout the experiments, with the cyanobacterium growth. The pure CO₂ was added at the bottom of the cultures in small bubbles and each individual addition lasted for 20 to 45 s. The pH of the culture medium was measured immediately before and after the addition of CO₂. This experimental condition is designed as “C+”;
- c) 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 cyanobacterium 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 five culture flasks ($n = 5$), exposed to $350 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ measured with a Biospherical Instruments quanta meter 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 $21 \pm 1^\circ\text{C}$. Salinity in the experiments was 33.0 psu. Growth was estimated based on daily microscopic cell counting with Neubauer chambers. Cultures were not buffered and pH was determined daily, at least at the beginning, in the middle and in the end of the light period. All samplings for cell counts occurred during the first 30 min of the light period. The pH measurements were also done at the beginning of the photoperiod and immediately before and after each manual addition of pure CO_2 in the C+ experiments. The initial cell densities of cultures were $2.0 \times 10^5 \text{ cell mL}^{-1}$. The experiments were carried out for 12 days. The volumes of the cells were measured using the equation provided by Hillebrand *et al.* (1999), assuming a spherical shape for the cyanobacterium. Mean volumes were based on measurements of 30 cells in each culture flask, giving five mean values used for statistics ($n = 5$).

Sampling procedure

Each culture was sampled twice for chemical analysis (on the 6th and 12th days of growth), in late-exponential and stationary growth phases (Fig. 1). Samples of 1.6 to 2.3 L were concentrated by centrifugation at $8,000 \times g$ for 9 min, at least once, 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 12 psu salinity. 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, 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 into Whatman GF/F[®] glass microfibre filters ($0.7 \mu\text{m}$ 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 cyanobacterium. 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 pigments. The filtered samples of culture media were kept at -18°C in polyethylene flasks until analysis.

Chemical analysis

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 and carotenoid were extracted in 90% acetone at 4°C for 20 h, after grinding the filters with the samples. Spectrophotometric determination of chlorophyll 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 analysis of variance (ANOVA) followed, where applicable, Tukey multiple comparisons, or by Student's *t*-test (Zar, 1996), in both cases adopting a level of significance $\alpha = 0.05$.

RESULTS

Growth curves of *Synechococcus subsalsus* in the three experiments are shown in Figure 1. Final cell yields were similar in the control and in the treatment with addition of pure CO_2 ($P > 0.05$). Cell densities at the end of the experiments were $5.23 \times 10^7 \text{ cell mL}^{-1}$ (control) and $5.43 \times 10^7 \text{ cell mL}^{-1}$ (C+). The treatment N- produced a higher final cell yield in comparison to the other experiments ($P < 0.05$), achieving 6.08×10^7

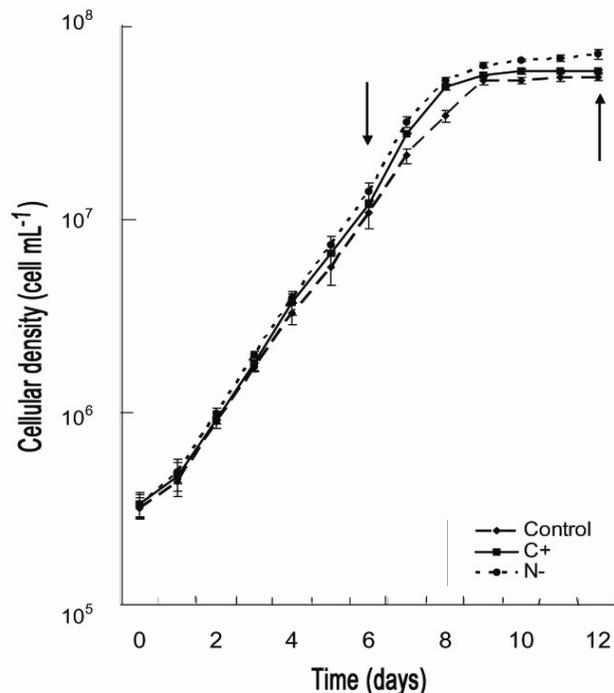


Figure 1. Growth curves of *Synechococcus subsalsus* cultured in three different experimental treatments: standardized conditions (control), with addition of pure CO₂ from the 5th day of growth (C+), and under nitrogen limitation from the 7th day of growth (N-). Arrows indicate the moments in which cells were sampled to perform chemical analyses. Each point in the curves represent the mean of five replicates \pm standard deviation (n = 5).

cell mL⁻¹. Similar growth rates were found in the three treatments throughout the exponential growth phases, with values >1.0 (with basis on log₂ of cell numbers) which generated increases of 2.0- to 2.5-fold of cell densities every 24 h. In the stationary growth phase rates were lower (typically <0.20), but slightly higher values were found in the N- experiment.

Fluctuations in cell biovolumes of *Synechococcus subsalsus* were found throughout the experiments. Average values of cell biovolumes are exhibited for exponential and stationary growth phases in Table 1. For the control, values recorded in the exponential growth phase were significantly higher than those found in the stationary growth phase ($P < 0.01$). For the treatments C+ and N- there was not significant variations of cell biovolumes in different growth phases ($P > 0.05$).

In both control and N- experiments, pH values fluctuated widely throughout the photoperiod. Measurements of pH at the start of the light period gave always lower values, typically ca. 8.2. The

Table 1. Measurements of cell volumes (μm^3) of the cyanobacterium *Synechococcus subsalsus* in two growth phases and three different experimental treatments: standardized conditions (control), with addition of pure CO₂ (C+), and under nitrogen limitation from the 7th day of growth (N-). Each value represents the mean of five replicates \pm standard deviation (n = 5). Mean values significantly different: * $P < 0.05$, a > b. The absence of letters indicates that mean values are not significantly different.

Growth phases	Experiments		
	Control*	C+	N-
Exponential	2.54 \pm 0.21 ^a	2.21 \pm 0.23	2.21 \pm 0.17
Stationary	2.26 \pm 0.13 ^b	2.27 \pm 0.19	2.02 \pm 0.15

values of pH increased throughout the photoperiod and achieved >9.1 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 8th day of growth) daily maximum values of pH were lower (typically <8.8) than in the exponential phase. In the C+ treatment the pH values were always kept below 8.5, even at the end of the photoperiod in the exponential growth phase.

There was a remarkable trend of decreasing the concentrations of nitrate and phosphate throughout the experiments (Figs. 2, 3). In the three experiments nitrate depletion was total by the end of the cultures. Nitrate exhaustion occurred in the 10th day in both C+ and N- treatments, and in the 11th day in the control. No experiment showed phosphate exhaustion, but more than 90% of the phosphate was taken up in both control and C+ experiments by the end of the cultivation (Fig. 3). In the experiment N- there was a peak of phosphate (~170 μM) in 6th day of growth, as a result of the addition of Conway nutrient solutions (without NaNO₃). A high concentration of phosphate (~140 μM) was still present in the culture medium at the end of the N- experiments. Variable concentrations of nitrite (0.2-2.9 μM) and ammonia/ammonium (0.5-3.6 μM) were also detected in all experiments (data not presented), but these values were negligible in comparison to the nitrate concentrations.

Values for protein, carbohydrate, lipid, chlorophyll-*a*, and total carotenoid are shown in Table 2. The protein content of *S. subsalsus* varied from 15.1 to 22.1% of the dry weight (dw). In the control, there was no significant variation of the protein content in different growth phases. On the other hand, in both C+ and N- experiments the protein content decreased from the exponential to the stationary growth phase.

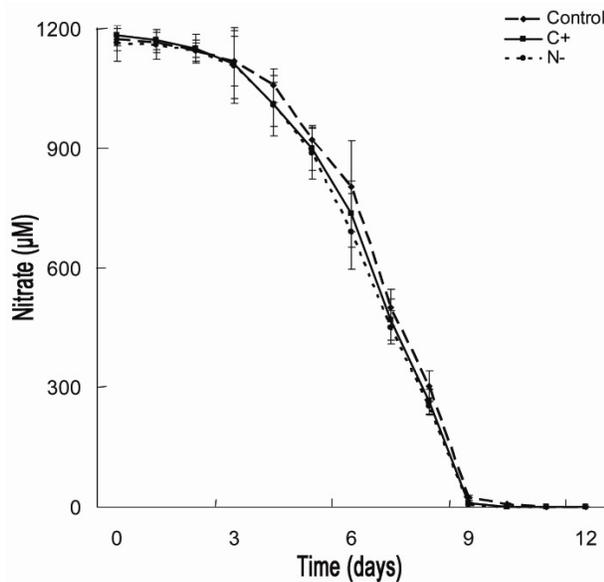


Figure 2. Variations of the nitrate concentrations in cultures of *Synechococcus subsalsus* cultured in three different experimental treatments: standardized conditions (control), with addition of pure CO₂ from the 5th day of growth (C+), and under nitrogen limitation from the 7th day of growth (N-). Values are expressed in micromolar concentration. Each point in the curves represent the mean of five replicates \pm standard deviation (n = 5).

In all experiments the carbohydrate content increased significantly from the exponential to the stationary growth phase (Table 2). In the exponential growth phase the carbohydrate content varied around 27% dw among the experiments, but the values were higher than 42% dw in the stationary growth phase. A similar trend was found for the lipid and total carotenoid contents: in all experiments the concentrations increased throughout growth. For lipid, typical concentrations were *ca.* 8.3% dw in the exponential growth phase and *ca.* 12.1% dw in the stationary growth phase. For total carotenoid, typical concentrations were *ca.* 0.14% dw in the exponential growth phase and *ca.* 0.20% dw in the stationary growth phase. Conversely, the concentrations of chlorophyll-*a* were higher in the exponential growth phase, achieving *ca.* 0.38% dw among the experiments, decreasing in the stationary growth phase to *ca.* 0.30% dw (Table 2).

DISCUSSION

Growth, cell biovolumes and nutrient uptake

Growth curves of *S. subsalsus* were similar in the treatments tested here. Growth rates higher than 1.0

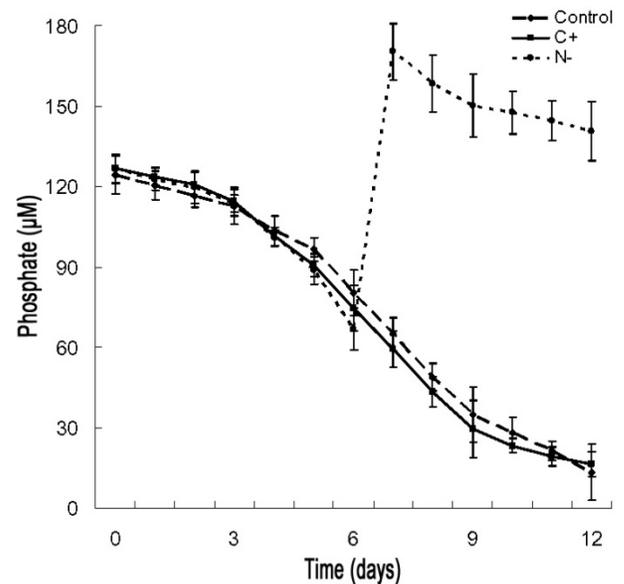


Figure 3. Variations of the phosphate concentrations in cultures of *Synechococcus subsalsus* cultured in three different experimental treatments: standardized conditions (control), with addition of pure CO₂ from the 5th day of growth (C+), and under nitrogen limitation from the 7th day of growth (N-). Values are expressed in micromolar concentration. Each point in the curves represent the mean of five replicates \pm standard deviation (n = 5).

recorded in the exponential growth phase suggest that the addition of pure CO₂ in C+ experiments did not promote a significant increase of biomass. Previous experiments performed by Lourenço *et al.* (2004) with 12 algal species recorded evidence of carbon limitation in all cultures run without inputs of aeration. Particularly, Lourenço *et al.* (2004) observed a significant greater final yield of *S. subsalsus* cultured with aeration in comparison to the treatment without aeration. Current results might mean that the input of CO₂ created by the use of aeration was enough to promote a fast growth of *S. subsalsus*, and the use of additional carbon as pure CO₂ was not effective to increase its biomass. A similar trend was found by Faria *et al.* (2012) in experiments run with the marine eustigmatophyte *Nannochloropsis oculata* with input of CO₂: the addition of excess of dissolved carbon did not promote a greater growth. This interpretation has also support in the study of Chiu *et al.* (2009), who found the highest productivity with cultures of *N. oculata* with small enrichment with carbon and either neutral or negative effects on productivity in treatments with high concentrations of pure CO₂. On the other hand, experiments with nitrogen starvation gave a higher final cell yield. This behavior may be related to the fact that many nutrients were added to

Table 2. Chemical profile of the cyanobacterium *Synechococcus subsalsus* in two growth phases and three different experimental treatments: standardized conditions (control), with addition of pure CO₂ (C+), and under nitrogen limitation from the 7th day of growth (N-). Values are expressed as percentages of substances in relation to the dry weight. Each value represents the mean of five replicates ± standard deviation (n = 5). 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.

Experiment/growth phase	Protein	Carbohydrate	Lipid	Chlorophyll- <i>a</i>	Total carotenoid
Control		***	*	*	*
Exponential phase	21.5 ± 1.9	28.0 ± 2.3 ^b	8.6 ± 0.7	0.40 ± 0.03 ^a	0.14 ± 0.02 ^b
Stationary phase	18.5 ± 2.4	42.5 ± 2.6 ^a	12.3 ± 1.3	0.32 ± 0.02 ^b	0.18 ± 0.02 ^a
C+	**	***	*	*	**
Exponential phase	22.1 ± 0.9 ^a	28.3 ± 2.7 ^b	8.5 ± 1.3	0.36 ± 0.03 ^a	0.13 ± 0.02 ^b
Stationary phase	17.4 ± 1.6 ^b	44.5 ± 2.4 ^a	11.9 ± 1.2	0.28 ± 0.03 ^b	0.20 ± 0.03 ^a
N-	*	***	*	*	***
Exponential phase	20.1 ± 2.0 ^a	25.8 ± 3.8 ^b	8.1 ± 0.9	0.39 ± 0.03 ^a	0.14 ± 0.02 ^b
Stationary phase	15.1 ± 1.4 ^b	47.3 ± 1.8 ^a	12.5 ± 1.3	0.31 ± 0.03 ^b	0.25 ± 0.02 ^a

the culture medium on the 7th day of growth, except nitrogen. We can hypothesize that the addition of metals and/or other components of the stock solutions of the Conway culture medium could have stimulated the cyanobacterium to grow more in comparison to the other treatments. We also recognize that small amounts of nitrogen were added with the use of the stock solutions of the Conway medium (in vitamins and (NH₄)₆MoO₂₄·4H₂O). However, we assume that this input of nitrogen was negligible, lower than 1.0 μM.

As all experiments exhibited similar growth rates in the exponential phase, differences in final cell yield among treatments derived from slight differences in growth rates recorded in the stationary phase. This trend is related to the fact that the experimental conditions were similar in the first half of all treatments. The addition of pure CO₂ occurred from the 5th day of growth in the treatment C+, and the addition of nutrients (except nitrogen) was done in the 7th day of the experiment N-. Other authors who dealt with different culture conditions since the beginning of experiments reported effects on growth rates starting in the exponential growth phase (e.g., Yu *et al.*, 2009; Zupo *et al.*, 2011).

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 high growth rate of *S. subsalsus*, a small-sized species. In the present experiments, cell biovolumes decreased from the exponential to the stationary growth phases only in the

control. This trend seems to be related to the progressive reduction of nutrient concentrations and low ability to accumulate energy reserves (carbohydrates and/or lipids) by the test species. Similar trends were reported by Lourenço *et al.* (1997) in experiments with *Tetraselmis gracilis*, a prasinophyte with low concentrations of lipid. In the other treatments there was no difference in average cell biovolumes between growth phases. The same trend was recorded by Faria *et al.* (2012) in cultures with *Chlorella* sp. and *Nannochloropsis oculata*, which showed no fluctuations of cell biovolumes throughout time/growth phases. We hypothesize that the addition of CO₂ in C+ experiments and nutrients in N- experiments might have contributed to avoid the trend of decreasing cellular biovolumes throughout time, as recorded in the control. The additions of CO₂ or dissolved nutrients seem to have ameliorated the physiological status of the cells in the stationary growth phase, keeping their typical biovolumes.

Daily variations of pH values in the experiments are explained by carbon uptake from the culture medium, carbon inputs, and photosynthetic activity (Goldman *et al.*, 1982; Rigobello-Masini *et al.*, 2003). The typical low values of pH at the start of the photoperiod are derived from the continuous input of aeration in the dark period without photosynthetic activity (Lourenço, 2006). The values of pH increased throughout the photoperiod as a result of active photosynthesis and uptake of dissolved carbon by the cells. After 11 h of light and CO₂ uptake with photosynthetic activity, pH values typically reached >9.1 in the control and in the N- treatment, depending on the cell density. In the C+ treatment, the addition of pure CO₂ kept the pH below 8.2, even at the end of

the photoperiod. These results surely indicate that the availability of carbon for the cyanobacterium was greater in the experiments with addition of CO₂ compared to the control.

The nitrate uptake was efficient in all experiments, which were finished under nitrogen exhaustion. This means that nitrogen deprivation was experimented in variable intensities in all treatments. At the 6th day of growth, nitrogen availability was ca. 65% of the initial concentrations in the culture medium at the start of the experiments (1.18 μM); however, by the 9th day of growth, less than 5% of the initial concentrations of nitrogen were still available to the cells, in a moment in which the cell densities were higher than 5.0x10⁷ cell mL⁻¹ (Fig. 1). In this context, it is coherent to assume that the stationary growth phase was established mainly by nitrogen limitation (Eriksen *et al.*, 2007). A second factor that might have contributed to the start of the stationary growth phase is light availability, considering the dark color recorded in the experiments (Johnsen & Sakshaug, 2007). The excess of cells possibly produced a self-shading in the cultures and a presumed effect of photoacclimation by *S. subsalsus* (Lourenço, 2006).

Phosphorus has not limited the growth of *S. subsalsus* in the present study. The phosphate concentrations declined throughout all experiments, but in none of them it was exhausted. In control and C+ experiments, the concentrations of phosphate in the 12th day of growth were ca. 13 and 16 μM, respectively, which represent more than 10% of the initial concentration of P added to the culture medium. Particularly in the treatment N-, final concentrations of dissolved P (ca. 140 μM) were higher than those recorded at the beginning of all experiments (128 μM). A phosphate peak (ca. 170 μM) observed on the sixth day of cultivation resulted from the addition of nutrient stock solutions of the Conway medium prepared without NaNO₃. 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 (LaRoche *et al.*, 1993). The expectation for the N- experiments is that ATP-dependent physiological processes would not be constrained by energy, considering the sufficiency in phosphorus. In addition, the lack of nitrogen would stimulate the cells to synthesize more lipid (*e.g.*, Rodolfi *et al.*, 2009; Li *et al.*, 2011).

Gross chemical composition

The protein content of *S. subsalsus* was low, fluctuating around 20% of the dry weight in all observations. Peak values for protein reported here are lower than those recorded by Lourenço *et al.* (2002),

who found higher values (>30%) for protein in *S. subsalsus* cultured in culture media rich in nitrogen (nitrate, ammonium or urea). Different culture conditions may account for significant changes in final results of protein, as stressed by Barbarino & Lourenço (2005). The protein production is favored in periods of suitable nitrogen availability, with limited carbohydrate synthesis. Conversely, during periods of reduced nitrogen availability there is an accumulation of carbohydrate and a decrease of protein production (Silva *et al.*, 2009). The lipid content also tends to increase under nitrogen limitation (Praveenkumar *et al.*, 2012). Higher values of protein were found in the exponential growth phase, under sufficiency in nitrogen (see the previous discussion on dissolved nutrients), decreasing in the stationary growth phase. The fast uptake of nitrogen and the high biomass produced in the current experiments did generate a physiological condition of nitrogen deprivation, confirmed by the analysis of dissolved nitrate in the culture medium. The low availability of nitrogen implicated a low protein content, which is in accordance with other authors (*e.g.*, Young & Beardall, 2003; Silva *et al.*, 2009). This interpretation is supported by the decrease in the concentrations of protein from the exponential to the stationary growth phases of C+ and N- experiments. This might mean that the experimental conditions tested in C+ and N- treatments were unsuitable for protein synthesis in the stationary growth phase. If this interpretation is correct, it is presumable that a further decrease in protein concentration could be found in case of longer essays (*e.g.*, 15-18 days of growth instead of the present 12 days).

Synechococcus subsalsus did show significant variations of carbohydrate content in all experiments, with a remarkable increase occurring from the exponential to stationary growth phase ($P < 0.001$). These data suggest a sufficiency in carbon throughout all experiments and also effects of nitrogen limitation contributing to increase the production of carbohydrate by this cyanobacterium. The synergistic coupling of carbon and nitrogen on the chemical composition of microalgae is well known in the literature (*e.g.*, Turpin, 1991; Geider & LaRoche, 2002), and the current results are in accordance with this knowledge. The increase of carbohydrate from the exponential to the stationary growth phase was 34.1, 36.4 and 45.5% for the control, C+ and N- respectively. High concentrations of carbohydrate were also found by Lourenço *et al.* (2002) in studies with *S. subsalsus* (24.8%), without induction of synthesis of non-protein substances. The higher contents of carbohydrate found here indicate that the cyanobacterium was induced to

produce these substances under the culture conditions selected to run the experiments.

Lipid content of microalgae may fluctuate widely (Rodolfi *et al.*, 2009), stimulated by factors as carbon and nitrogen availability (Suali & Sarbatly, 2012). Genetic and phylogenetic traits are influential, but many authors use to test different culture conditions searching to increase the production of lipid (*e.g.*, Dempster & Sommerfeld, 1998; Suali & Sarbatly, 2012). In the present experiments, *S. subsalsus* exhibited variations of lipid concentrations with the same trend recorded for carbohydrate: increase from the exponential to the stationary growth phases. The increase of carbohydrate from the exponential to the stationary growth phases was 30.1, 28.64 and 35.2% for the control, C+ and N- respectively. Despite the increments of lipid throughout time were significant the values themselves are relatively low, which point to restrictions for the use of this species if high concentrations of lipid are needed. This trend seems to be typical of cyanobacteria. Selvan *et al.* (2013) recorded 10.4 and 9.2% of total lipid (dw) for *Synechococcus* sp. and *Lyngbya* sp., respectively, under nitrogen starvation. Afify *et al.* (2010) found a similar value, 9.2% dw of lipid for the filamentous cyanobacterium *Spirulina platensis*. There are many reports in the literature of eukaryotic microalgae that responded to nitrogen starvation with expressive increases in lipid production (Lombardi & Wangersky, 1995; Guevara *et al.*, 2005; Chen *et al.*, 2011; Karatay & Dönmez, 2011), contrasting with our study.

Variations in concentrations of chlorophyll and total carotenoid showed opposite trends. Chlorophyll decreased from the exponential to the stationary growth phase, and this trend is probably related to the limitation of nitrogen. Chlorophyll is a nitrogenous substance and its synthesis is influenced by the availability of nitrogen (Lourenço *et al.*, 1997; Silva *et al.*, 2009). The carotenoid contents increased from the exponential to the stationary growth phase in the three experiments. Carotenoids are not influenced by the availability of nitrogen, since they are not composed by this element and microalgae can keep synthesizing carotenoids even under nitrogen starvation (Grünewald *et al.*, 1997; Hanagata & Dubinsky, 1999). In addition, high densities of cells may stimulate the production of carotenoids due to the self-shading of the cultures (Johnsen & Sakshaug, 2007).

Potential for biodiesel production and conclusions

Microalgae present variable chemical compositions and their chemical characteristics can be changed depending on the experimental conditions used to increase the production of biomass or specific

substances of interest (Lourenço, 2006; Spolaore *et al.*, 2006).

Growth rates of *Synechococcus subsalsus* were high, pointing to potentially relevant biomass productivity in larger systems of cultivation. The results also suggest that the main form of accumulation of energy reserve in the species tested here occurs through carbohydrate, not lipid. This is consistent with information about the phylogeny of the group to which it belongs -Cyanophyta (Graham *et al.*, 2009). In the current experiments we used the strategy of culturing *S. subsalsus* in two steps, the first one plenty of nutrients and the second one under depleted nitrogen or abundance of carbon. However, the cyanobacterium did not show more efficiency to uptake nutrients in different treatments and the biomass was similar among the experiments. The most remarkable effect recorded here was the increase in the carbohydrate content throughout time.

The addition of pure CO₂ did not cause a significant increase in the biomass of *S. subsalsus*, so it was not useful in our experiments. The nitrogen starvation of the cultures caused an intense accumulation of carbohydrate, but increments in production of lipid were minor. These results indicate that *S. subsalsus* has a virtually null potential for biodiesel production, given its low concentrations of lipid. Despite the concrete perspectives of development of better photobioreactor systems that could improve the production and use of microalgae (*e.g.*, Pulz, 2001; Kwon *et al.*, 2012), this is unlikely to be enough to increase such low concentrations of lipid (*ca.* 10-12% dw) to a level of viable use as a feedstock for biodiesel production (*e.g.*, Schenk *et al.*, 2008; Francisco *et al.*, 2010; Rajvanshi & Sharma, 2012). Other species with better chemical profiles would be more appropriate to be selected. The high concentrations of carbohydrates point to the potential use of this species as raw material for other possible biotechnological processes such as the generation bioethanol, which viability still has to be demonstrated. The promise of genetically modified microalgae (*e.g.*, Henley *et al.*, 2013; Wijffels *et al.*, 2013) could meet organisms like *S. subsalsus*, which has high growth rates, but presents an unsuitable chemical composition for biodiesel production.

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