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Low light intensity and nitrogen starvation modulate the chlorophyll content of *Scenedesmus dimorphus*

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Running title: Light and nitrogen – dependent modulation of *S. dimorphus* chlorophyll

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Abstract

Aims: Chlorophyll is a photosynthetic pigment found in plants and algal organisms and is a bioproduct with human health benefits and a great potential for use in the food industry. The chlorophyll content in microalgae strains varies in response to environmental factors. In this work, we assessed the effect of nitrogen depletion and low light intensity on the chlorophyll content of the *Scenedesmus dimorphus* microalga.

Method and results: The growth of *S. dimorphus* under low light intensity led to a reduction in cell growth and volume as well as increased cellular chlorophyll content. Nitrogen starvation led to a reduction in cell growth and the chlorophyll content, changes in the yield and productivity of chlorophylls *a* and *b*. Transmission electron microscopy was used to investigate the ultrastructural changes in the *S. dimorphus* exposed to nitrogen and light deficiency.

Conclusions: In contrast to nitrogen depletion, low light availability was an effective mean for increasing the total chlorophyll content of green microalga *S. dimorphus.*

Significance and impact of study: The findings acquired in this work are of great biotechnological importance to extend knowledge of choosing the right culture condition to stimulate the effectiveness of microalgae strains for chlorophyll production purposes.

Keywords: *Scenedesmus dimorphus*; Nitrogen; Light intensity; Chlorophyll; Cell volume.

Introduction

Microalgae are fast-growing photosynthesizing organisms that require light for their growth, which may be from sunlight, as well as carbon dioxide, water and some essential inorganic nutrients, such as phosphorus and iron (Chisti 2007; Hlavová *et al.* 2015). Due to the culture under more controlled and optimized conditions, photosynthesis in microalgae is more efficient than in vascular plants (reviewed by Pirt 1986); hence, they possess a higher rate of biomass production. Additionally, microalgae tolerate diverse climates and do not need fertile land (Huang *et al.* 2010). In the field of biotechnology, microalgae have great potential as source of bio-products for use in the food, pharmaceutical and cosmetic industries (Borowitzka, 1992). Microalgae are a key unicellular resource of commercially important protein, lipids, carotenoids, enzymes, vitamins, minerals, phenolic compounds, polysaccharides and chlorophyll (Gouveia *et al.*, 2010).

Chlorophyll is a vital active photosynthetic natural pigment widely distributed in plants and algal organisms (Humphrey 1980). In addition to its biological importance, chlorophyll has great potential as a biotechnological product, where it is already used as natural food coloring (Timberlake and Henry 1986) and has health benefits in the fields of cosmetology, making part of products such as shampoos, deodorants and facial and body moisturizers (Ahad, 1994), and dermatology, due to its effectiveness in dermatitis treatment (Cady and Morgan 1948). Chlorophyll and its derivatives have been studied as physiologically active components of the human diet. In fact, chlorophyll and its derivatives, the pheophytins and pheophorbins, have antioxidant and antimutagenic activities *in vitro* (Ferruzzi *et al*. 2002), and Negishi and co-workers (1997) showed the antigenotoxic activity of chlorophyll in *Drosophila*. Moreover,

chlorophyll derivatives produce antiproliferative effects in human colorectal adenocarcinoma cells (Vesenick *et al.* 2012). In human studies, chlorophyll and its derivatives were decreased the cancer risk induced by mutagens present in the diet (Jubert *et al.* 2009; Egner *et al.* 2011).

The use of microorganisms to obtain chlorophyll is both more productive and faster compared with plants, because the cultivation of microorganisms allows evaluation in a continuous manner, exhibits faster growth and is not seasonal (Chauhan and Pathak 2010). In microalgae, the chlorophyll content varies in response to physical factors, such as light intensity, agitation and temperature, and chemical factors, such as nutrient availability. In *Chlorella vulgaris*, temperatures above 40 °C produced a decrease in chlorophyll content (Chinnasamy *et al.* 2009). In *Scenedesmus obliquus*, increasing the agitation led to a reduction in chlorophyll amount due to more uniform light distribution (Martínez *et al.* 2000). Variation in nutrient availability *in vitro* can alter the chlorophyll concentration in cultures; for example, reduced magnesium concentration in *Chlorella vulgaris* cultures leads to a decrease in the chlorophyll concentration (Finkle and Appleman 1953). Low nitrogen concentrations in *Chlamydomonas reinhardtii* and *Scenedesmus subspicatus* cultures also lead to the reduction of chlorophyll content (Dean *et al.* 2010). The amount of chlorophyll also varies with the stage of cultivation, as it is often reduced in stationary phase (Fidalgo *et al.* 1998).

Because chlorophyll is a potential biotechnological product from microalgae and its content is related to photosynthetic efficiency and cell growth, it is important to understand how changing the culture conditions modulate chlorophyll production and other cellular effects. Specifically, the influence of nitrogen stress and light intensity need to be described in detail,

because they are the conditions known to alter the cellular content of chlorophyll. Herein, we investigated the potential of microalgae cultivation for chlorophyll production in an important microalga strain by monitoring the effects of nitrogen depletion and low light intensity on the production of chlorophyll by *Scenedesmus dimorphus*. In addition, other simultaneous cellular responses were evaluated as a function of these changes in the culture conditions, including cell volume, cell growth and lipid production.

Materials and methods

Microalgae: The green microalga *Scenedesmus dimorphus* (UTEX 1237) was obtained from the Culture Collection of Algae of the University of Texas (UTEX) at Austin, USA. Microalgae were grown in 250 mL Erlenmeyer flasks containing 100 mL of ASM-1 medium (0.17 g L^{-1} $NaNO_3$; 0.041 g L^{-1} $MgCl_2.6H_2O$; 0.049 g L^{-1} $MgSO_4.7H_2O$; 0.029g L^{-1} $CaCl_2.2H_2O$; 17.4 mg L^{-1} K_2HPO_4; 35.6 mg L^{-1} $Na_2HPO_4.12\ H_2O$; 2.48 mg L^{-1} H_3BO_3; 0.39 mg L^{-1} $MnCl_2.4H_2O$; 1.08 mg L^{-1} $FeCl_3.6H_2O$; 0.335 mg l^{-1} $ZnCl_2$; 19µg L^{-1} $CoCl_2.6H_2O$; 1.4 µg L^{-1} $CuCl_2$ and 7.44 mg L^{-1} EDTA Na_2, pH 6.5) (Gorham *et al.* 1964) at 25 °C, in a light/dark ratio of 12:12 hours, under a light intensity of 123.47 ± 8.23 µmol photons/m^{-2} s^{-1} (control condition) as measured by a Heinz Walz Gmbh luminance meter equipped with a sensor (s/n: SQSA0404). All experiments were performed in triplicate. To evaluate the effect of the absence of nitrogen on the chlorophyll content of *S. dimorphus*, the source of nitrogen ($NaNO_3$) was completely removed from the ASM-1 medium. To assess the effect of reduced light intensity, the microalgae were cultured at a mean light intensity of 16.91 ± 0.45 µmol photons/m^{-2} s^{-1}.

Cell growth: Microalgal densities were determined by measuring the optical density of the algal cultures at 600 nm (OD_{600}) for 0, 3, 7 and 14 days of growth. To obtain the ratio of cell density per optical density, dilutions were prepared (1:1, 1:2, 1:10 and 1:100) with a raw aliquot of 7 x 10^6 cells ml^{-1}. Each diluted aliquot was counted in triplicate in a Neubauer chamber, and the absorbance was read in triplicate at 600 nm in a Biotek Synergy 2 spectrophotometer. To estimate cell density, the results were analyzed by the straight-line equation, obtained from a linear regression of optical density versus cell count. The relationship obtained between microalgal density (D, cells ml^{-1}) and OD_{600} is shown, with a correlation coefficient $r^2 = 0.9989$, by the following equation: $D = (OD_{600} - 0.0301)/ 9 \times 10^{-8}$. This method has a strong positive correlation between cell concentration and OD_{600} ($P < 0.0001$, Pearson's correlation; confidence interval of 95%). Growth curves were constructed using the GraphPad Prism 5 software to evaluate differences between the treatments.

Analysis of chlorophyll content: Chlorophyll analysis was performed using the trichromatic method according to the Standard Methods for the examination of water and waste water (Eaton *et al.* 1995). Briefly, *S. dimorphus* cultures were concentrated using 13 mm glass fiber prefilters (Sartorius and Swinnex® Millipore). Subsequently, the sample-containing filters were macerated in a mixer homogenizer (OMNI International) with 90% acetone. The samples were transferred to glass tubes in a final volume of 3 mL of 90% acetone and were kept in the dark at 4 °C for 24 hours. Then, the samples were centrifuged (500 x *g* for 5 minutes), and the supernatant was evaluated in a spectrophotometer (Lambda 25, PerkinElmer) at 664 and 647 nm to determine the concentration of chlorophyll *a* and chlorophyll *b*, respectively. In order to correct the turbidity effect, the values of the absorbances at 664 and 647 nm were subtracted

from the value at 750 nm. The chlorophyll concentration was calculated at 0, 3, 7 and 14 days from the sum of the concentrations of chlorophylls *a* and *b*, extrapolated to a 1 m^3 sample and the productivity was then calculated from the concentration (mg m^{-3}) divided by the time in days.

Morphometric analysis: The microalgae cultivated in different culture conditions were collected by centrifugation (1160 x *g* for 5 minutes) and then washed in ASM-1 medium. Cells were allowed to adhere for 20 minutes onto glass coverslips previously coated with 0.1% poly-L-lysine. Then, the samples were washed in ASM-1 medium, and bright field images were acquired with an optical microscope (Leica DM5000B). The images were used to measure the width and length of the microalgae using the Axio Vision Rel 4.8 software. One hundred cells were measured for each culture condition in three independent experiments. The cell volume was estimated by $V = \pi a2b/3$, where a is the length and b is the width (Chen *et al*. 2011).

Transmission Electron Microscopy (TEM): For TEM analysis samples were prepared according to Solomon *et al*., (1986). Briefly, *S. dimorphus* cultivated for 14 days were fixed in 2.5% glutaraldehyde, 4% paraformaldehyde, 0.25 M sucrose in 0.1M cacodylate buffer, pH 7.3, for 2 h at room temperature, washed twice in 0.25 M Sucrose in 0.1 M cacodylate buffer, pH 7.3 and postfixed in 1% osmium tetroxide, 2% potassium ferrocyanite, 0.25 M Sucrose in 0.1 M cacodylate buffer, pH 7.2 for 45 min in the dark, dehydrated in an acetone series (30%, 50%, 70%, 90% and 100%) and embedded in low viscosity embedding media Spurr's Kit, according to manufacture's instructions. Ultrathin sections were obtained Leica EM UC ultramicrotome and were observed in a Tecnai Spirit Biotwin G^2 transmission electron microscope operating at 80 kV.

Statistical analysis: To evaluate the growth curves, fluorimetry and morphometry, Student's T test was used, comparing each experimental group with the control group. To evaluate the chlorophyll content analysis, two-way ANOVA test with Bonferroni post-test was used. For all analyses, a 95% confidence interval was used in the statistical tests performed with GraphPad Prism 5.

Results

Effect of nitrogen absence and low light intensity on cell growth

The effect of nitrogen absence (-N) and low light intensity (-L) on *Scenedesmus dimorphus* growth, in comparison with the control condition (C), is depicted in Fig. 1. All culture conditions showed a gradual increase in cell density, even for those with low light intensity and those grown in the absence of nitrogen. Stationary phase was reached at day 14 (Fig. 1A). The microalgae incubated in the control condition attained the highest cell density, reaching 8.05 x 10^6 cells mL^{-1} on day 14, whereas the (-N) group reached 3.6 x 10^6 cells mL^{-1} and the (-L) group reached 3.08 x 10^6 cells mL^{-1} on the same day. There was a clear negative relationship between the growth rate and the nitrogen- and light-limited culture conditions (Fig. 1A). The calculation of the area under the growth curve (Fig. 1B) revealed that, in comparison with control cells, the (-N) and (-L) groups had a significant reduction in cell densities by 45.85% and 52.18%, respectively. The (-L) culture condition exhibited the highest anti-proliferative effect on *S. dimorphus*, yielding 11.68% less than the (-N) group. An additional experiment was done conjugating nitrogen starvation and low light intensity (-NL) to investigate the effect on cell growth (Fig. S1). This combined condition exhibits a strong cell growth inhibition compared to

control cell in all time points analyzed. However, when compared with both (-N) and (-L), the (-NL) group presented significant difference in cell density only on day 14.

Changes in chlorophyll content in response to low light and N absence

The images of *S. dimorphus* grown for 14 days at three different culture conditions were obtained by light microscopy. The green cellular pigment related to chlorophyll was clearly reduced in microalgae grown in (-N) cultures and increased in cells grown in (-L) cultures when compared with the control condition (Fig. 2A).

To examine whether the (-N) and (-L) groups were able to alter the chlorophyll content per cell, a time course analysis was performed (Fig. 2B). In the control cells, a non-significant variation in chlorophyll content was observed until 7 days of culture, and then an abrupt reduction by 34.43% of the chlorophyll quantity was found between days 7 and 14. Cells grown in low light intensity showed no temporal decrease in the amount of chlorophyll, whereas the N starvation led to gradual decrease, comprising a final loss of 77.53% in the cell chlorophyll content compared with the initial time. Compared with the control group, the (-N) group presented a 39.54% reduction in the content of chlorophyll after 3 days of culture. On days 7 and 14, decreases of 56.05% and 36.10% were found, respectively. Conversely, under light limitation, the chlorophyll content was similar to the control group through 7 days of culture. However, on day 14, the concentration of chlorophyll per cell in the (-L) group was remarkably higher than the control group, achieving a difference of 128.09%.

The chlorophyll productivity was also evaluated, and the control group showed a significant decrease until day 14 (Fig. 2C). Both the (-N) and (-L) groups had lower productivities than the control from day 3 to 14, where the (-N) group had a decrease of 40.22% on day 3, 21.57% on day 7, and 27.29% on day 14. However, the (-L) group had decreases of 59.16%, 38.89% and 81.86% on the same respective days of culture. Therefore, the productivity of all groups decreased significantly within the growth period analyzed.

The cellular concentration of chlorophylls *a* (Chl *a*) and *b* (Chl *b*) relative to the total chlorophyll per cell was investigated for 14 days as a function of culture conditions (Fig. 2D). In the control condition, Chl *a* was present at the highest level, comprising approximately 81% of the total chlorophyll of *S. dimorphus*, whereas the Chl *b* content were 19%. The percentage of Chl *a* remained unchanged within the time examined for the control and (-L) groups. However, the (-N) group presented a decrease of approximately 8.77% in Chl *a* content after 14 days of culture. The percentage of Chl *b* remained unchanged in the control and low light groups, but an increase by approximately 42.21% was measured after 14 days in the (-N) group.

Analysis of cell size and volume

To evaluate whether there was variation in the *S. dimorphus* shape during the absence of nitrogen and in low light intensity, the cell size (Fig. 3A) and volume (Fig. 3B) were measured. The control group showed a mean height of 14.26 ± 1.99 µm, a mean width of 4.23 ± 0.72 µm and a mean volume of 941.70 ± 375.20 μm^3. The (-N) group presented a width 10.62% greater than the control cells, whereas a 5.39% reduction was found in the height. For the (-L) group, the width and the height were 15.56% and 10.09% smaller than the control cells, respectively. The

cell volume of the (-L) group was 31.67% smaller than the control cells, but there was no volume variation found in the (-N) group.

Analysis of nitrogen absence and low light intensity on the ultrastructure of *S. dimorphus*

The ultrastructural changes caused by low light intensity and nitrogen depletion on *S. dimorphus* were determined by TEM analysis. An extensive analysis of ultrathin sections revealed that both culture conditions remarkably changed the ultrastructure of *S. dimorphus*. Fig. 4A, B show the overall structure of *S. dimorphus* from 14-day-old culture in the control condition. In this stage, *S. dimorphus* presented small lipid bodies (LBs) spread on the cell cytoplasm and the photosynthetic thylakoids were not prominent and were randomly arranged. The starch granules were found in low amount in the intrathylakoid space. Electron-dense granules resembling phosphate granules were also seen. In (-N) group, it was readily observed that the LB number and size increased greatly (Fig. 4C, D). An enhanced amount of starch granules was also found in the microalgae exposed to N-limitation. Apparently, such culture condition led to a reduction on the size of chloroplasts. In contrast, in the (-L) group the size of chloroplasts increased tremendously compared to both control and (-N) groups (Fig. 4E, F). As result, the main structural feature was the appearance of a large number of chloroplast thylakoid membranes. Interestingly, in the cell cytoplasm either lipid bodies or starch granules were rarely visualized.

Discussion

The modification of culture conditions for microorganisms typically results in changes in cell growth and biomass production or composition (Kilham *et al.* 1997; Liu *et al.* 2008; Nigam *et al.* 2011). Nitrogen availability and light intensity are among the key factors that modulate the microalgal growth and biochemical composition, and likely the chlorophyll content. Light supply is a major limiting factor for autotrophic microalgal growth (Rubio *et al*. 2003) and nitrogen is essential for both protein and nucleic acids synthesis, and is also found in chlorophyll molecules (Lourenço *et al.* 1998). As a consequence, nitrogen availability and light intensity are crucial for microalgae growth and division, and also for photosynthesis. Although the variance of nitrogen and light intensity has been fully investigated on neutral lipid accumulation in oleaginous microalgal species, including *Scenedesmus* sp. (Ho *et al.* 2012; Liu *et al.* 2012; Ruangsomboon *et al.* 2013), their influence on chlorophyll content had not yet been described in detail. In this work, the *Scenedesmus dimorphus* green microalgae was used to assess the effect of light limitation and nitrogen starvation on chlorophyll production. In addition, the effects of these conditions on cell growth, cell size, cell volume and lipid accumulation were examined.

The reduction of light intensity has been demonstrated to be an important factor in decreasing the growth of these microorganisms (Danesi *et al.* 2004). Nutrients present in the culture medium are also important for microalgae growth. For example, the cultivation of microalgae in nitrogen-limited conditions has led to a significant decrease in the cell density (Dean *et al.* 2010; Nigam *et al.* 2011). Our results indicated that low light intensity as well as nitrogen restriction reduced *S. dimorphus* growth. However, the anti-proliferative effect was more pronounced in cells cultivated under light limitation.

For algae, modifications of their photosynthetic response to light have an adaptive significance by maximizing their potential growth. Microalgae grown in low light (1) perform less photosynthesis to generate less energy and (2) use this available energy to produce a more efficient light-capturing system, but their chloroplasts consequently expand. These adaptations, called light-shade adaptations, involve a two- to ten-fold increase in the chlorophyll content. Cells at high light intensities expend less of their resources on chlorophyll synthesis and more on the enzymes related to carbon fixation, such as in the synthesis of RuBP carboxylase and possibly in the photosynthetic dark reactions (Darley 1982; Carvalho *et al.* 2011). *S. dimorphus* investigated in this work when exposed to light-limited conditions, make light-shade adaptations including a huge increase in thylakoid membrane and an increase in their chlorophyll content to capture light in a more efficient manner.

Nitrogen reduction in cultured microalgae could be harmful for cell growth, because this element is a main component of proteins, nucleic acids and chlorophyll (Lourenço *et al.* 1998). We demonstrated that *S. dimorphus* cultivated in the control condition exhibited a significant reduction in the chlorophyll content in the late stage of culture (from 7 to 14 days). This corroborates previous studies carried out with the *Isochrysis galbana* green microalga, where the amount of chlorophyll was reduced at the stationary phase (Fidalgo *et al.* 1998). Furthermore, we demonstrated that the absence of nitrogen in the culture medium significantly reduced the chlorophyll production of *S. dimorphus*. The (-N) group had a gradual chlorophyll reduction over all evaluated culture days, which indicates that nitrogen availability is determinant for chlorophyll production. Chlorophyll is a molecule that contains a ring with four pyrrole groups and in its center; a magnesium atom is stabilized by the four pyrrolic nitrogen atoms (Humphrey,

1980; Wilkinson *et al.* 1990). Because the nitrogen atoms are part of the structure of chlorophyll, it is possible that the absence of nitrogen in the culture medium has a negative impact in the synthesis of chlorophyll, justifying the reduced chlorophyll content found in the N-limited cultures of *S. dimorphus*. In addition, the TEM analysis showed a reduction in the thylakoid membrane amount and an increase in body lipids in N-limited cultures. Similar results were visualized using *Chlorella minutissima*, which after three days under nitrogen starvation presented enhanced quantity of lipid bodies and condensed chloroplasts, which suggest a chloroplast damage caused by nitrogen starvation (Wang *et al.* 2011). The authors also showed by TEM analysis the concurrent increase in the number of LB and starch granules as result of N-limitation. This phenomenon was attributed to a process of energy partitioning between starch and lipid biosynthesis carried out in green microalgae when exposed to nitrogen deficiency. In the current work, similar result was visualized in the *S. dimorphus* ultrastructure.

Microalgae cultured in light-limited conditions showed a chlorophyll concentration per cell similar to the control group through day 7. On day 14, the (-L) group maintained its cellular chlorophyll concentration, whereas the control group underwent a reduction in chlorophyll content. This increase in chlorophyll concentration caused by low light intensity has been shown in the *Spirulina platensis* microalgae (Danesi *et al.* 2004) and in vascular plants, such as *Fagus sylvatica* (Minotta and Pinzauti 1996). This higher chlorophyll content seems to be a compensatory mechanism activated during inadequate light intensity to efficiently absorb and utilize light. On the order hand, previous works showed an elevation of chlorophyll concentration when light intensity was increased in the culture of *Ankistrodesmus falcatus, Nannochloropsis sp.* and *Chlorella sp* (Cheirsilp and Torpee, 2012; George et al. 2014). This is

indicative that the effect of light intensity on the chlorophyll content is species specific. However, it would be interesting to further investigate different microalgae species.

The chlorophyll productivity in the control, (-N) and (-L) groups was also evaluated. The most productive condition was the control, especially on day 3. On days 7 and 14, the control group presented a decrease in productivity from the reduction in the cellular concentration of chlorophyll over the course of the experiments. Similarly, this reduction in productivity of chlorophyll was observed in the (-N) and (-L) groups. As expected, the (-N) group had the lowest productivity, which is most likely due to the lowest cellular chlorophyll concentration. However, the increment of nitrogen concentration in the microalgae culture medium leads to the increase the growth rate and chlorophyll level (Piorrec et al. 1984, Li et al. 2008), ultimately increasing the chlorophyll productivity. The (-L) group, despite its increased chlorophyll concentration, had lower productivity compared with the control group, which may be explained by the lower biomass production in the light-limited culture conditions. In this sense, the two-stage method, which consists of initial cultivation in high light intensity to enhance the cell growth with subsequent low light intensity cultivation to favor the chlorophyll content, seems to be a promising strategy to increase the chlorophyll productivity in microalgae. Danesi et al (2004) working with two-stage method achieved an increase of up to 29 % in *Spirulina platensis,* when compared with culture condition at fixed light intensity. Interestingly, microalgae are capable of producing the highest level of cellular chlorophyll content under photoautotrophic growth when compared with heterotrophic and mixotrophic cultures (Cheirsilp and Torpee, 2012). This result strongly indicates that the light availability plays a major role on the chlorophyll production and accumulation in microalgae.

The cellular chlorophylls were evaluated using the trichromatic method (Eaton *et al.* 1995), which is based on the spectrophotometric reading of chlorophylls *a* and *b* per cell using their absorption peaks. In the control condition, *S. dimorphus* had mainly chlorophyll *a*, comprising approximately 81% of the total chlorophyll, whereas chlorophyll *b* averaged 19% of the total. We also observed that the proportion of the different types of chlorophyll remained unchanged over time for the cells grown in low light intensity. In contrast, cells grown in the N-depleted medium had altered proportions of the different types of chlorophyll, achieving a final composition of 75.51% Chl *a* and 24.49% Chl *b*. Therefore, this indicates that the proportion of different types of chlorophyll may be altered in different culture conditions. The change in the ratio of different chlorophyll types in microalgae grown under nitrogen depletion may be the result of a higher level of degradation of chlorophyll *a* and *b*. Li et al., (2008) hypothesized that during the nitrogen deficiency in the culture medium of microalgae, the chlorophyll, which contain 4 nitrogen atoms per molecule, is consumed as a way to recycling the intracellular pool of nitrogen allowing the cell growth.

Cellular morphology modification induced by change in culture medium is generally observed, and this includes microalgae (Donk e*t al.* 1997; George *et al.* 2004; Pancha *et al.* 2014). In the current work, we followed the specific *S. dimorphus* volume and size response to N- and L-limited conditions. Nitrogen removal from the culture medium of *S. dimorphus* led to changes in the cell volume. Kilham and co-workers (1997) showed a significant reduction in the cell volume of *Ankistrodesmus falcatus* grown in N-limited medium. The authors attributed this phenomenon to the low concentration of proteins found in cells grown in these conditions. In contrast, Donk and colleagues (1997) found a significant increase in the cell volume of

Chlamydomonas reinhardtti and *Selenastrum capricornatum*. Similar results were found in *Chlamydomonas reinhardtii* and *Scenedesmus subspicatus* by Dean and co-workers (2010), where the increased cell volume in microalgae cultivated under N limitation was attributed to the enhancement of intracellular lipid bodies, which are a type of energy reserve. In this work, we showed that *S. dimorphus* grown in absence of nitrogen resulted in an increase in cell width and a decrease in its height. Thus, there was no change in volume compared with the control group. One possible explanation for this finding is that the increase in the accumulation of lipids may have been offset by a reduction in the amount of proteins, which is frequently observed in microalgae cultures without nitrogen (Dean *et al.* 2010; Piorreck *et al.* 1984). However, cells grown in low light intensity showed a decrease in height as well as width, which implies in a reduction in the cell volume. These results are in agreement with the results reported by Thompson *et al.* (1991), who showed that luminosity is directly related to the cell volume. The mechanisms of these changes are still unclear; however, it is known that microalgae can vary cell volume in response to internal ion concentration or variations in the molecular weight of photosynthetic metabolites.

Understanding the behavior of *S. dimorphus* in the specific culture conditions investigated in this study may be a strategy to make it economically feasible for use in different biotechnological applications, such as microalgal chlorophyll production. The results obtained in this investigation can provide information to guide further researchers. These findings also represent an initial step towards the development of other studies regarding the modulation of the production of unicellular photosynthetic microalgae biomass as a source of chlorophyll.

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Conflict of interest

No conflict of interest declared.

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Figure Legends

Figure 1: Fig. A - Growth of *S. dimorphus* in control (C, gray circle), nitrogen deprived (-N, white circle) and low light (-L, black circle) conditions. **Fig. B** - Area under the growth curve of *S. dimorphus* control (C), nitrogen deprived (-N) and low light (-L) conditions. The experiments were performed in triplicate and a confidence interval of 95% was used. * $P < 0.05$ *** $P < 0.0001$.

Figure 2: Fig. A - Light microscopy images of *S. dimorphus* cultured in the absence of nitrogen (-N), in the control condition (C) and at low light intensity (-L). **Fig. B** - Cell chlorophyll content in *S. dimorphus* grown in control (C), nitrogen deprived (-N) or low light intensity (-L) conditions. The experiments were performed in triplicate and a confidence interval of 95% was used. * $P < 0.05$ *** $P < 0.0001$ relative to the control group. **Fig. C** - Productivity of chlorophyll in *S. dimorphus* grown in control (C), nitrogen deprived (-N) or low light intensity (-L) conditions. *** $P < 0.001$. **Fig. D** - Average percentage of chlorophyll *a* and *b* relative to the total chlorophyll content of *S. dimorphus* under control (C), nitrogen deprived (-N) and low light intensity (-L) conditions. *** $P < 0.0001$.

Figure 3: Measuring the width and length (**Fig. A**) as well as cell volume (**Fig. B**) of *S. dimorphus* cultured under control (C), nitrogen deprived (-N), and low light intensity (-L) conditions. ** $P < 0.01$ *** $P < 0.0001$. **Fig. C** - Evaluation of lipid production by *S. dimorphus* cells cultured in control (C), nitrogen deprived (-N) and low light intensity (L) conditions from the mean fluorescence intensity of cells stained with Nile Red. * $P < 0.05$.

Figure 4: Transmission electron microscopy images of *S. dimorphus* cultured in control, nitrogen-deprived and low light intensity conditions. **Fig. A –** Longitudinal section showing general morphology of *S. dimorphus* cultivated in control condition, where it is possible to observe the typical arrangement of cellular organelles. **Fig. B** - Higher magnification of squared region shown in **Fig. 4A**. **Fig. C, D –** Ultra-thin sections of *S. dimorphus* exposed to nitrogen-deprived condition showing the presence of numerous large lipid bodies and starch granules. **Fig. E –** General view of the ultrastructure of *S. dimorphus* cultivated under low light intensity condition showing the cell cytoplasm almost filled with chloroplasts. Note the higher number of thylakoid membranes. Neither lipid bodies nor starch granules are observed in this representative image. **Fig. F –** Higher magnification of image of *S. dimorphus* region showing in detail the arrangement of chloroplast thylakoid membranes. S – starch granules, L – lipid bodies, Tk – thylakoid membranes, Py – pyrenoid; PG – phosphate granule.

Supporting Information Legend

Supporting Information 1 - *S. dimorphus* grown in control (C, white circle), nitrogen deprived (-N, light gray), low light (-L, black circle) as well as in nitrogen deprived and low light condition (-NL). The experiments were performed in triplicate and a confidence interval of 95% was considered. *** $P < 0.001$ relative to the control group and a = $P < 0.05$, (-NL) relative to (-N) and (-L) groups.