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Biochemical and physiological responses of *Selenastrum gracile* (Chlorophyceae) acclimated to different phosphorus concentrations

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Abstract

Algae are able to adjust their metabolism according to their environment, maximizing growth rate and production of biomolecules under adverse conditions such as pulses of excess of a contaminant or limitation of a nutrient. In order to evaluate the effects of phosphorus (P) availability on the biochemical composition of the freshwater microalga *Selenastrum gracile*, we acclimated the microalgae to different phosphorus concentrations. After acclimation, exponentially growing cells were inoculated and after 120 h, samples were processed for the determination of carbohydrate, lipid, fatty acid, chlorophyll, cell density, growth rate, and dry weight. Cell density, growth rate, and dry weight decreased with less P, while chlorophyll *a*, carbohydrates, lipids, and fatty acids per cell increased under P limitation. According to our lipid class and fatty acid results, algae alter their metabolism and membrane configuration to avoid more structural or metabolic damage under limitation, especially at 23 μmol P L^{-1}. The most sensitive parameters under P limitation were chlorophyll *a*, lipids, and poly- and monounsaturated fatty acids. The changes in fatty acids contributed to the fluorescence and photosynthesis changes under P limitation, and they occurred before changes were detected in other parameters, such as growth rate. Furthermore, we suggest that prior acclimation to different P affected microalgal physiology and metabolism.

Keywords Acclimation · Biomolecules · Fatty acids · Lipids · Nutrient limitation · Photochemical efficiency

Introduction

Algae require sufficient nutrients for their healthy metabolism, with phosphorus (P) being of great importance due to its role in energy metabolism and in nucleic acids (Beardall et al. 2005; Dyhrman 2016). Phosphorus limitation affects cell division, chlorophyll *a* production, and photosynthesis (Cembella et al. 1984, Alcoverro et al. 2000), as well as the synthesis of organic molecules such as proteins, carbohydrates, and lipids (Guschina and Harwood 2006, Lai et al. 2011), and it is usually limiting for primary producers in freshwater environments (Elser et al. 2013).

Algae can capture more phosphorus than needed for their metabolism (luxury uptake) and store it as polyphosphate bodies (Fogg 1973). These phosphorus reserves are important for maintaining metabolism during some cell divisions in a changing environment (Bhola et al. 2011) and contribute to algae resistance to metals that bind to the polyphosphate granules (Verma et al. 1993). Altering P at the base of the trophic chain can affect consumers and higher trophic levels (DeMott and Van Donk 2013), and some species are able to regulate their physiology or biochemistry in response to phosphorus scarcity (Van Mooy et al. 2009, Wu et al. 2012). Under replete conditions, proteins can constitute up to 60% of cell mass (Geider and La Roche 2002), but under nutrient limited conditions, there are usually more carbohydrates and lipids than proteins (Kilham et al. 1997, Bertilson et al. 2003), and these changes can be transferred to other trophic levels, affecting the food webs (Hartwich et al. 2012).

When the medium or the environment is P-scarce, there is an impairment and an adjustment of algal metabolism

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(Beardall et al. 2005; Touzet et al. 2007), resulting in a decrease in photosynthesis and growth (Alcoverro et al. 2000) and in the biochemistry and fatty acid composition of algae (Spijkerman and Wacker 2011). Some algal species are able to regulate and adjust their machinery to new P conditions (Wu et al. 2012; Rocha et al. 2016a). In addition, algae can optimize their uptake according to changes in concentrations of limiting nutrients (Smith and Yamanaka 2007).

Photosynthesis can be affected under stress, e.g., a decrease in the quantum yield (Ritter et al. 2014), and be correlated with the inhibition of algal growth (Magnusson et al. 2012) and changes in synthesis of chlorophyll *a* under nutrient limitation. Phosphorus is present in enzymes and co-factors that act in photosystems I and II—PSI and PSII—(e.g., NADP, G3P—glycerate 3-phosphate, and ATP), being fundamental to photosynthesis reactions (Reynolds 2006), which can be reflected in changes of the maximum quantum yield (F_v/F_m) of PSII (Krause and Weis 1991).

To evaluate the effects of phosphorus limitation on *Selenastrum gracile*, a common freshwater microalga in aquatic tropical and subtropical oligotrophic ecosystems, we acclimated the alga to two phosphorus concentrations lower than the control's P concentration. Then, we analyzed the biochemical composition, growth, chlorophyll *a* content, and some photosynthetic parameters (maximum and operational quantum yield, photochemical and non-photochemical quenchings), after the acclimation process to evaluate how the P deficiency affects its metabolism. Our hypothesis is that exponentially growing cells acclimated at different P concentrations will adjust their physiology and biochemistry to minimize the effects of reduced P supply.

Methods

The freshwater microalga *Selenastrum gracile* (Reinsch) (CH 005) was obtained from the algae culture collection of the Botany Department at the Federal University of São Carlos (São Carlos, SP, Brazil). Stock cultures were kept in LC Oligo medium (AFNOR 1980), which has the following composition: $Ca(NO_3)_2.4H_2O$ (1.7×10^{-4} M), KNO_3 (1.0×10^{-3} M), $MgSO_4.7H_2O$ (1.2×10^{-4} M), K_2HPO_4 (2.3×10^{-4} M), $CuCl_2.H_2O$ (6.0×10^{-8} M), $(NH_4)_6Mo_7O_{24}.4H_2O$ (2.4×10^{-8} M), $ZnSO_4.7H_2O$ (1.0×10^{-7} M), $CoCl_2.6H_2O$ (1.3×10^{-7} M), $Mn(NO_3)_2.H_2O$ (1.5×10^{-7} M), $C_6H_8O_7.H_2O$ (1.4×10^{-7} M), H_3BO_3 (4.9×10^{-7} M), $C_6H_5FeO_7.H_2O$ (3.1×10^{-6} M), $FeCl_3.6H_2O$ (1.9×10^{-6} M), $FeSO_4.7H_2O$ (1.1×10^{-6} M), and $NaHCO_3$ (1.8×10^{-4} M). The algae were cultured at pH 7.0 under controlled conditions of light intensity (150 μmol photons m^{-2} s^{-1}), photoperiod (16:8 h light:dark cycle), and temperature (22 ± 2 °C). Every 12 h, the cultures were gently shaken manually. Culture media were sterilized through autoclaving for 20 min at 121 °C before inoculation. Although cultures were not axenic, sterile conditions were used throughout and only sterile materials were used in culture manipulation. Laboratory materials were washed with neutral detergent and kept for 7 days in 10% HCl before use.

Phosphorus (P) was furnished as K_2HPO_4 at the following concentrations: 230—control (C), 23 (10%), and 2.3 μmol L^{-1} (1% of control). The P concentration in the control was that present in the LC Oligo medium. These concentrations were chosen after preliminary tests, where algae were exposed to a range from 10^{-2} to 10^4 μmol P L^{-1}, and we observed inhibition of growth and photosynthesis in concentrations ≤ 1 μmol P L^{-1} and ≥ 10^3 μmol P L^{-1}. Since the aim was to characterize changes in growth, photosynthesis, and biochemistry, we avoided using concentrations where photosynthesis and growth were inhibited. Microalgal cell acclimation was performed using semi-continuous cultures. The algae in exponential growth phase were inoculated at an initial cell density of 10^5 cells mL^{-1} in three experimental replicates in 500 mL polycarbonate Erlenmeyer flasks containing 200 mL of sterile culture medium, and after 4 days in exponential growth, we started the partial renewal of culture medium. This was performed every 72 h, keeping cell density around 2×10^5 cells mL^{-1}, the density present in mid-exponential growth phase. Using this time interval, the algae were always kept in the exponential phase during the acclimation. The amount of fresh medium added varied according to the cell density. Cells were taken every 24 h and counts were performed under an optical microscope using a Neubauer-improved chamber to determine the growth rate. Specific growth rates were obtained by plotting the natural log of cells per milliliter versus time and calculating a linear regression for the exponential growth, as described by Rocha et al. (2015).

Intracellular phosphate can sustain algal growth during few cell divisions under P limitation (Bhola et al. 2011). In our study, cells were kept at a specific P concentration for several weeks, allowing the determination of seven growth rates until at least three of them were not significantly different ($p > 0.05$). Then, algae were considered acclimated and their metabolism reflecting the external P concentration, i.e., the P available in the culture medium. Dissolved orthophosphate content in culture media was determined employing the ascorbic acid method (APHA 1995).

These acclimated cells were then inoculated at exponential growth phase in fresh medium in three experimental replicates in 500 mL polycarbonate Erlenmeyer flasks containing 200 mL of sterile culture medium. Cells were inoculated providing an initial cell density of approximately 10^5 cells mL^{-1} and biochemical analyses were done after 120 h. Preliminary tests showed that cells were in the exponential growth phase

and this time was sufficient to provide enough biomass for the biochemical analysis.

To determine chlorophyll *a*, samples (10 mL) were filtered onto cellulose ester membranes (0.45 μm pore size—Millipore), the filter was transferred to a Falcon tube, and 5 mL of DMSO was added to each tube (Shoaf and Lium 1976). Samples were kept in the dark for 45 min and optical measurements were made in a spectrophotometer (HACH DR 5000, USA) at 664 and 647 nm wavelengths. Blanks were performed using a clean filter submitted to the same extraction procedure. Chlorophyll *a* concentration (pg cell^{-1}) was calculated as described by Jeffrey and Humphrey (1975).

Dry weight was determined on previously baked glass fiber filters (400 °C for 24 h) which were cooled to room temperature in a desiccator and weighed (Sartorius MC21S, ± 1 μg). One hundred milliliter of algal cultures was filtered and filters were kept at 60 °C in an oven until constant weight.

Biochemical analysis

All glassware was first washed with tap water and neutral detergent, then rinsed with tap water and placed in 10% HCl for 7 days, after which it was rinsed with deionized and ultrapure water. Glass fiber filters (GF/C; Boeco, Germany) were previously baked at 400 °C for 24 h and the glassware used for lipid analysis was baked at 400 °C for 12 h and rinsed with methanol and chloroform just before use.

Lipid classes

Total lipids and lipid classes were extracted and measured by thin layer chromatography with flame ionization detection (TLC/FID) using an Iatroscan MK6 (Mitsubishi Kagaku Iatron Inc., Japan) according to Parrish (1999). Culture samples (100 mL) were filtered onto glass fiber filters which were then ground with a metal rod, and lipid extractions were done in chloroform:methanol:chloroform extracted water (2:1:1), with 5 min sonication (Unique Group, Indaiatuba, Brazil) and 2 min centrifugation at 3000 rpm (Eppendorf 5702R, Germany). The organic layer containing the lipids was removed and transferred to a vial; then, 6 mL of chloroform was added and the procedure was repeated three times. Samples were concentrated under ultrapure N_2, sealed and stored at − 20 °C until analysis. For the TLC chromatography, samples (and standards) were spotted onto quartz rods (Chromarod SIII) using a Hamilton syringe. The samples were focused twice in 100% acetone and placed in a constant humidity chamber for 5 min. Three solvent systems were used for the complete sample development that resulted in the detection of nine lipid classes. The first solvent system was composed of hexane:diethyl ether:formic acid (98.95:1:0.05), the second was hexane:diethyl ether:formic acid (79:20:1), and the third was chloroform:methanol:chloroform extracted water (5:4:1). After each development, the rods were kept in the Iatroscan for 5 min before scanning and for 5 min in a humidity chamber after scanning. The analytical conditions for the FID runs were hydrogen flow 173 mL min^{-1}, air flow 2 L min^{-1}, and scan speed 4 mm s^{-1}. Lipid classes were quantified using calibration curves made with lipid standards obtained from Sigma-Aldrich (USA). In addition, diacylglycerol *N*-trimethylhomoserine (DGTS) was purchased from Avanti Polar Lipids, Inc. (USA) to verify that this betaine lipid eluted with phospholipids in the last development system.

Fatty acid methyl esters

Fatty acid methyl ester (FAME) derivatives were prepared using 14% boron trifluoride in methanol (BF_3/CH_3OH) and hexane (Morrison and Smith 1964, Budge and Parrish 1998). The lipid extract was dried under nitrogen and 0.5 mL hexane and 1.5 mL 14% BF_3/CH_3OH were added. The mixture was shaken, sonicated for 4 min, topped with nitrogen, and heated at 85 °C for 1.5 h. The sample was cooled to room temperature, 0.5 mL chloroform extracted water was added and then 2 mL hexane, forming two layers. The upper, organic layer was removed and the tube was flushed with nitrogen, capped, sealed with Teflon tape, and stored at − 20 °C until analysis.

FAME analysis was performed on a HP 6890 GC FID equipped with a 7683 autosampler. The GC column was a ZB wax+ (Phenomenex, USA). The column length was 30 m with an internal diameter of 0.32 mm. The column temperature began at 65 °C where it was held for 0.5 min. The temperature ramped to 195 °C at a rate of 40 °C min^{-1}, held for 15 min, then ramped to a final temperature of 220 °C at a rate of 2 °C min^{-1}. This final temperature was held for 0.75 min. The carrier gas was hydrogen flowing at 2 mL min^{-1}. The injector temperature started at 150 °C and was ramped to a final temperature of 250 °C at a rate of 120 °C min^{-1}. The detector temperature stayed constant at 260 °C. Peaks were identified using retention times from standards purchased from Supelco: 37 component FAME mix (product number 47885-U), bacterial acid methyl ester mix (product number 47080-U), PUFA 1 (product number 47033), and PUFA 3 (product number 47085-U). Chromatograms were integrated using the Varian Galaxie Chromatography Data System, version 1.9.3.2.

Total carbohydrates

Total intracellular carbohydrates were determined using the modified phenol-sulfuric method according to Liu et al. (1973). Samples (10 mL) were centrifuged at 1500 rpm for 10 min (Eppendorf 5702R, Germany); the supernatant was discarded and the pellet was used for the determination of carbohydrates. The pellet was resuspended with distilled water (0.5 mL) and 1 mL 10% phenol; thus, the samples were

shaken and 5 mL H_2SO_4 was added. During 10 min, the samples were kept in a tray containing ice and, after this period, the samples were centrifuged for 10 min at 4400 rpm, the supernatant was removed and placed in a cuvette, and the absorbance at 485 nm was measured against blank reagent (HACH DR 5000; HACH Company, USA). Carbohydrates quantification was based on calibration curves using glucose as standard.

Photochemical efficiency

The maximal photosynthetic efficiency (Φ_M) and the parameters related to photosynthetic decay were determined in a pulse-modulated fluorometer Phyto-PAM (Walz, Germany). Erlenmeyers with algal culture were gently shaken manually and *ca* 3 mL were taken up and dark-adapted for 15 min, to open the chlorophyll reaction centers. After this adaptation, we measured the initial fluorescence (F_0) and maximum fluorescence (F_m) and the difference between these two parameters provided the variable fluorescence (F_v). Using F_v and F_m values, the maximum photosynthetic efficiency of PSII (F_v/F_m) was obtained. The quantification occurred during 6 days of culture in exponential phase.

After determining these parameters at 72 h (day 4), when the algae were in the mid-exponential phase, light adaptation was induced. The samples were exposed to continuous actinic light (128 μmol photons m^{-2} s^{-1}) and new saturating light pulses were applied every 20 s—for 15 min—to obtain the parameters of light-adapted samples, where there is the closure of reaction centers. The steady-state chlorophyll fluorescence (F_s) and maximum fluorescence in light $\left(F'_m\right)$ allowed the calculation of the operational quantum yield (($\Phi' = (F'_m - F_s)/F'_m$)), photochemical quenching (qP), non-photochemical quenching (qN), and Stern-Volmer non-photochemical quenching (NPQ) using Eqs. 1, 2 (Juneau et al. 2002), and 3 (Maxwell and Johnson 2000), respectively:

$$qP = \left(F'_m - F_o\right)/\left(F'_m - F'_o\right) \quad (1)$$

$$qN = 1 - \left[\left(F'_m - F'_o\right)/(F_m - F_o)\right] \quad (2)$$

$$NPQ = \left(F_m - F'_m\right)/F'_m \quad (3)$$

Data analysis

Statistical analyses were based on ANOVA and Tukey's post hoc test at $p < 0.05$. The data were obtained from three experimental replicate cultures and are presented as mean ± SD of the replicates. A correlation matrix-based principal component analysis (PCA) was used to determine the relationship between analyzed parameters.

Results

In P-limited cultures, the cell density decreased but the growth rate during the exponential growth phase was not affected at 23 μmol P L^{-1}, but just at the most limiting P concentration (2.3 μmol L^{-1}). Also, we observed a decrease in dry weight (mg L^{-1}) with less P; however, there were no differences in dry weight per cell (≈ 15 pg $cell^{-1}$). There were no differences in total carbohydrates between control and 23 μmol P L^{-1}; however, at the lowest concentration (2.3 μmol P L^{-1}), the amount was more than two times higher ($p < 0.05$). Content of chlorophyll *a* per cell increased with less P, being higher than control, especially in the 23 μmol P L^{-1} treatment (Table 1).

The amount of lipids per cell increased significantly under P limitation compared to the control ($p < 0.05$). Hydrocarbons (HC), free fatty acids (FFA), and phospholipids (PL) + betaine lipids (BL) increased with the decrease in P available ($p < 0.05$). Ketones (KET) and the acetone mobile polar lipids (AMPL) increased at 23 μmol P L^{-1} ($p < 0.05$), however, without differences between control and 2.3 μmol P L^{-1} treatments. Triacylglycerols (TAG) decreased with less P available ($p < 0.05$), with the lowest value at 23 μmol P L^{-1}. Steryl ester/wax ester (SE/WE) and sterols (ST) were not affected by different P concentrations. Aliphatic alcohol (ALC) was detected only at the lowest concentration (2.3 μmol P L^{-1}) (Table 2). Besides the changes in amount of lipids per cell, the percentage of some lipid classes also changed under P limitation, e.g., KET increased

Table 1 Cell density (× 10^6 cells mL^{-1}), growth rate (d^{-1}), dry weight (mg L^{-1}), carbohydrate (pg $cell^{-1}$), and chlorophyll *a* (pg $cell^{-1}$) of *Selenastrum gracile* grown for 120 h in different phosphorus concentrations. C refers to control. Values are means ± SD for $n = 3$. Columns with the same superscript letters are not significantly different ($p > 0.05$)

Phosphorus (μmol L^{-1})	Cell density (× 10^6 cells mL^{-1})	Growth rate (d^{-1})	Dry weight (mg L^{-1})	Carbohydrate (pg $cell^{-1}$)	Chlorophyll *a* (pg $cell^{-1}$)
2.3	1.42 ± 0.05^c	0.88 ± 0.03^b	21.4 ± 1.30^b	9.60 ± 0.54^b	0.62 ± 0.04^c
23	2.05 ± 0.14^b	1.00 ± 0.07^a	27.5 ± 2.90^b	4.70 ± 0.43^a	1.27 ± 0.11^b
230 (C)	3.07 ± 0.25^a	1.14 ± 0.09^a	48.3 ± 1.60^a	4.70 ± 0.72^a	0.37 ± 0.02^a

Table 2 Lipid classes (pg cell^{-1}) of *Selenastrum gracile* grown for 120 h in different phosphorus concentrations. HC (aliphatic hydrocarbon); SE/WE (steryl ester/wax ester); KET (ketone); TAG (triacylglycerols); FFA (free fatty acids); ALC (aliphatic alcohol); ST (sterol); AMPL (acetone mobile polar lipids); PL (phospholipids); BL (betaine lipids). C refers to control. ND, not detected. Values are means ± SD ($n = 3$). Lines with same superscript letters are not significantly different ($p > 0.05$)

	Phosphorus (μmol L^{-1})		
Lipid class	2.3	23	230 (C)
HC	0.11 ± 0.02^{a}	0.07 ± 0.01^{ab}	0.04 ± 0.01^{b}
SE/WE	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
KET	0.01 ± 0.00^{b}	0.04 ± 0.01^{a}	0.01 ± 0.00^{b}
TAG	0.41 ± 0.02^{b}	0.27 ± 0.03^{c}	0.48 ± 0.01^{a}
FFA	0.31 ± 0.07^{a}	0.07 ± 0.01^{b}	0.06 ± 0.01^{b}
ALC	0.02 ± 0.00	ND	ND
ST	0.16 ± 0.02	0.13 ± 0.04	0.11 ± 0.01
AMPL	0.10 ± 0.02^{b}	0.24 ± 0.05^{a}	0.12 ± 0.02^{b}
PL + BL	0.91 ± 0.10^{b}	1.22 ± 0.09^{a}	0.47 ± 0.03^{c}
Total lipid	2.19 ± 0.09^{a}	2.08 ± 0.12^{a}	1.29 ± 0.06^{b}

from ≈ 0.5% at 2.3 and 230 μmol P L^{-1} to 1.8% at 23 μmol P L^{-1}, while FFA increased from ≈ 4% at 23 and 230 μmol P L^{-1} to 14% at 2.3 μmol P L^{-1}, and AMPL decreased from ≈ 10% at 23 and 230 μmol P L^{-1} to 5% at 2.3 μmol P L^{-1}. However, the most interesting and remarkable changes were observed for TAG and PL which eluted together with BL. While TAG significantly decreased from 37% (230 μmol P L^{-1}) to 13% at 23 μmol P L^{-1} and 24% at 2.3 μmol P L^{-1}, PL + BL were significantly higher at 23 μmol P L^{-1} (59%) compared to 230 μmol P L^{-1} (37%) and 2.3 μmol P L^{-1} (42%). HC (≈ 5%), SE (≈ 0.4%), and ST (≈ 7%) were almost constant independent of the P available.

Different patterns of response in fatty acid (FA) production were observed, such as a linear decrease with decreasing P (24:0), or a linear increase with decreasing P (16:0, 20:5ω3, sum of total FA and sum of saturated FA—SAFA). The 18:0, 20:3ω6, and the sum of monounsaturated FA (MUFA) had a slight decrease at 23 μmol P L^{-1}, while the sum of polyunsaturated FA (PUFA) and ω3 FA was the highest at this treatment (Table 3).

In terms of proportions, the fatty acids and groups shown in Table 4 were the most affected under P limitation. The percentages of MUFA and FA 18:1ω11, 20:1ω9, and 24:0 decreased with the increase in P limitation. The lowest values of

Table 3 Fatty acid composition (mg g dry weight^{-1}) of *Selenastrum gracile* grown for 120 h in different phosphorus concentrations. Σ Sat—sum of saturated fatty acids; Σ MUFA—sum of monounsaturated fatty acids; Σ PUFA—sum of polyunsaturated fatty acids; Σ ω3—sum of ω3 fatty acids; ?—tentative identification based on the literature. Values are means ± SD for $n = 3$. Lines with same superscript letters are not significantly different ($p > 0.05$)

	Phosphorus (μmol L^{-1})		
Fatty acid	2.3	23	230 (C)
16:0	23.8 ± 4.7^{a}	18.5 ± 3.8^{ab}	14.5 ± 1.0^{b}
18:0	1.6 ± 0.2^{a}	1.0 ± 0.1^{b}	1.2 ± 0.2^{ab}
18:1ω11?	24.0 ± 3.5^{a}	7.9 ± 0.7^{b}	15.1 ± 0.4^{c}
18:1ω7	0.8 ± 0.1^{a}	0.5 ± 0.2^{a}	0.0 ± 0.0^{b}
18:2ω6	4.8 ± 0.7	4.0 ± 0.6	2.8 ± 1.2
18:2ω4	0.6 ± 0.0^{a}	0.1 ± 0.0^{b}	0.1 ± 0.0^{b}
18:3ω3	11.9 ± 2.6^{b}	28.3 ± 6.2^{a}	9.8 ± 0.1^{b}
18:4ω3	2.8 ± 0.3^{ab}	4.1 ± 1.2^{a}	1.1 ± 0.3^{b}
18:4ω1?	1.0 ± 0.1^{a}	0.3 ± 0.2^{b}	0.0 ± 0.0^{b}
20:1ω9	2.3 ± 0.7^{a}	0.8 ± 0.1^{b}	1.4 ± 0.4^{ab}
20:1ω7?	4.4 ± 1.5^{a}	1.0 ± 0.6^{b}	0.6 ± 0.1^{b}
20:2ω6	0.4 ± 0.0^{a}	0.1 ± 0.0^{b}	0.1 ± 0.0^{b}
20:3ω6	3.1 ± 0.3^{a}	1.4 ± 0.5^{b}	2.0 ± 0.3^{b}
20:4ω6	1.0 ± 0.3^{a}	0.6 ± 0.0^{b}	0.2 ± 0.0^{c}
20:5ω3	0.6 ± 0.1^{a}	0.2 ± 0.0^{b}	0.1 ± 0.1^{b}
21:5ω3?	1.0 ± 0.1^{a}	0.7 ± 0.1^{a}	0.1 ± 0.0^{b}
24:0	0.5 ± 0.0^{c}	1.0 ± 0.1^{b}	2.3 ± 0.1^{a}
Sums	101.4 ± 11.7^{a}	85.0 ± 11.9^{ab}	61.2 ± 5.0^{b}
Σ SAFA	31.2 ± 5.0^{a}	24.9 ± 4.3^{ab}	20.2 ± 0.8^{b}
Σ MUFA	36.5 ± 3.6^{a}	14.6 ± 1.2^{c}	22.6 ± 3.2^{b}
Σ PUFA	32.5 ± 5.2^{ab}	42.3 ± 6.8^{a}	19.2 ± 0.3^{b}
Σ ω3	19.7 ± 4.4^{b}	34.5 ± 7.0^{a}	12.2 ± 0.3^{b}
C_{18} essential PUFA (18:3ω3 + 18:2ω6)	16.7 ± 3.3^{a}	32.3 ± 6.7^{b}	13.3 ± 0.2^{a}

Table 4 Fatty acid composition (% of total fatty acids) of *Selenastrum gracile* grown for 120 h at different phosphorus concentrations. Σ MUFA—sum of monounsaturated fatty acids; Σ PUFA—sum of polyunsaturated fatty acids; Σ ω3—sum of ω3 fatty acids; ?—tentative identification based on the literature. Values are means ± SD for $n = 3$. Rows with the same superscript letters are not significantly different ($p > 0.05$)

	Phosphorus (μmol L^{-1})		
Fatty acid	2.3	23	230 (C)
18:1ω11?	15.7 ± 0.6^{b}	9.3 ± 0.6^{c}	24.7 ± 2.4^{a}
18:3ω3	23.0 ± 2.6^{b}	33.0 ± 2.9^{a}	15.4 ± 0.8^{c}
20:1ω9	0.5 ± 0.3^{c}	1.0 ± 0.1^{bc}	2.2 ± 0.8^{a}
24:0	1.2 ± 0.1^{b}	1.1 ± 0.1^{b}	3.8 ± 0.3^{a}
Σ MUFA	26.1 ± 3.4^{b}	17.3 ± 2.2^{c}	36.9 ± 4.2^{a}
Σ PUFA	44.1 ± 1.9^{a}	49.7 ± 1.1^{a}	30.2 ± 2.2^{b}
Σ ω3	32.2 ± 0.7^{b}	40.4 ± 2.9^{a}	19.3 ± 0.7^{c}
C_{18} essential PUFA (18:3ω3 + 18:2ω6)	28.9 ± 2.8^{b}	37.7 ± 2.8^{a}	20.9 ± 1.5^{c}

MUFA and 18:1ω11 were obtained at 23 μmol P L^{-1}, while the 20:1ω9 was lowest at 2.3 μmol P L^{-1}. On the other hand, the FA 18:3ω3 and sums of ω3 FA and C_{18} essential PUFA increased with P limitation and the highest percentages were observed at 23 μmol P L^{-1}, differing from all other treatments. The sum of PUFAs increased under P limitation, and the values in all treatments were higher than the control ($p < 0.05$) and without differences between treatments ($p > 0.05$) (Table 4).

The maximal quantum yield of *Selenastrum gracile* at 72 h had a slight increase at 23 μmol P L^{-1}, with the highest values and a slight decrease at 2.3 μmol P L^{-1} compared to control. The operational yield decreased only at 2.3 μmol P L^{-1}, with the lowest values ($p < 0.05$) (Fig. 1).

We observed a slight decrease in qP (white bars) of *Selenastrum gracile* after 72 h growing in different P available in the medium. The non-photochemical (qN; gray bars) and Stern-Volmer non-photochemical quenching (NPQ; black bars) increased with the decrease of P available ($p < 0.05$), with the highest values at 2.3 μmol P L^{-1} treatment (Fig. 2).

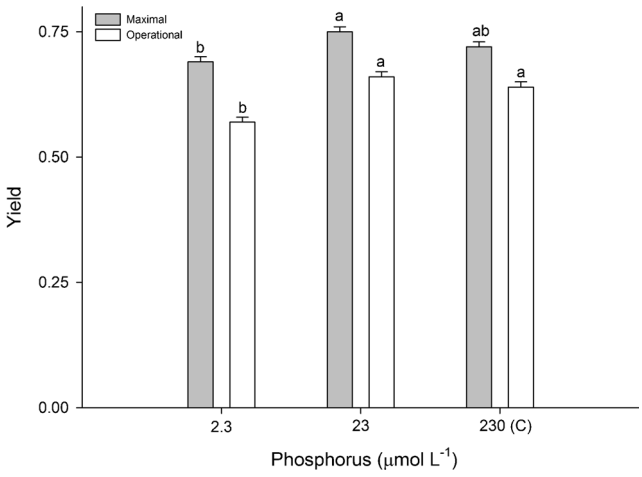

Fig. 1 Maximum PSII (gray bars) and operational quantum yield (white bars) of phosphorus-acclimated *Selenastrum gracile* grown for 72 h in different phosphorus concentrations. Different letters indicate significant difference ($p < 0.05$). Values are means ± SD ($n = 3$)

The PCA showed that the first two components were responsible for 77% of the total data variation, with a positive correlation of TAG, ST, and cell density in the control. The sums of SAFA, total lipids, total FA, and non-photochemical parameters (qN and NPQ) as well of FFA, ALC, MUFA, and carbohydrates, were negatively correlated with phosphorus supply, with samples of 2.3 μmol P L^{-1} being grouped close to the FFA, MUFA, ALC, and carbohydrate vectors, while samples from the intermediate concentration (23 μmol P L^{-1}) were grouped close to PL + BL, C18 Ess PUFA, and chlorophyll *a* (Fig. 3).

Discussion

Prior acclimation of *S. gracile* to P limitation allowed the microalgae to adjust their metabolism to face different conditions of P available in the medium in an attempt to maintain

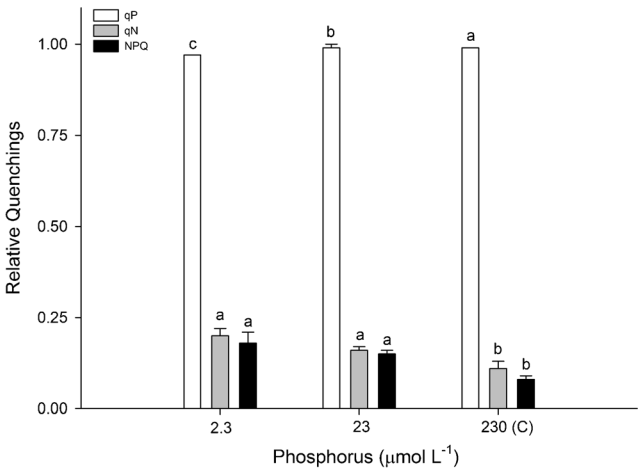

Fig. 2 Photochemical quenching (qP, white bars), quenching processes not related to photochemistry (qN, gray bars), and non-photochemical quenching, mostly related to heat dissipation (NPQ; black bars) of phosphorus-acclimated *Selenastrum gracile* after 72 h growing at different phosphorus concentrations. Bars with same color without a letter in common are statistically different ($p < 0.05$). Values are means ± SD ($n = 3$)

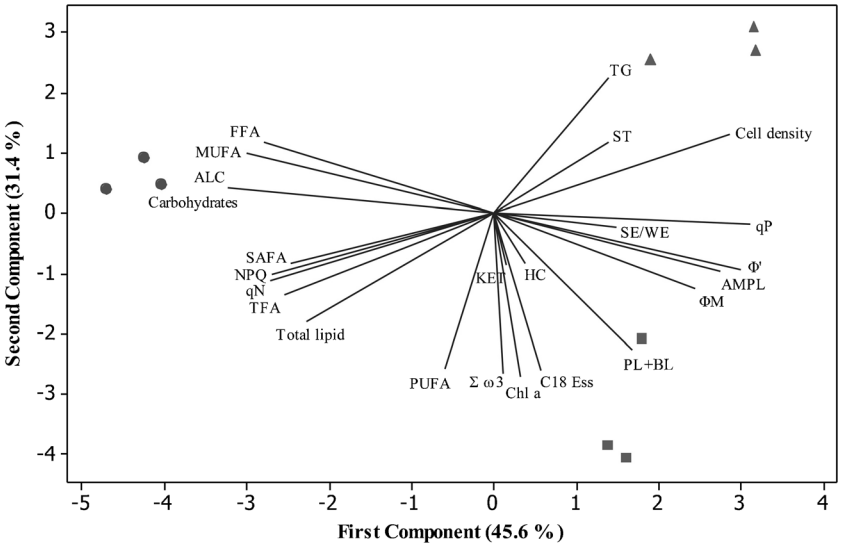

Fig. 3 PCA biplot showing the relationship of biochemical parameters (total lipids, carbohydrates, fatty acids), physiological (cell density and chlorophyll *a*), and photosynthetic parameters (quantum yields and quenchings) of phosphorus-acclimated *Selenastrum gracile* grown for 120 h in different phosphorus concentrations. HC (aliphatic hydrocarbon); SE/WE (steryl ester/wax ester); KET (ketone); TAG (triacylglycerols); FFA (free fatty acids); ALC (aliphatic alcohol); ST (sterol); AMPL (acetone mobile polar lipids); PL + BL (phospholipids and betaine lipids); PUFA (sum of polyunsaturated fatty acids); MUFA (sum of monounsaturated fatty acids); SAFA (sum of saturated fatty acids); Σ ω3 (sum of omega 3 fatty acids), C18 Ess (C_{18} essential polyunsaturated fatty acids); TFA (total fatty acids); Φ_M (maximum photochemical efficiency); Φ' (operational photochemical efficiency); qP (photochemical quenchings); qN and NPQ (non-photochemical quenchings). Triangles refer to control (230 μmol P L^{-1}), squares to 23 μmol P L^{-1}, and circles to 2.3 μmol P L^{-1} treatments

structure, photosynthesis, and growth. This highlights the importance of acclimation when evaluating the effects of P limitation and the value of chemical measurements combined with growth and photosynthetic parameters in eliciting the algal response to limitation. Under nutrient limitation, algae reduce their investment in growth and produce reserve compounds with high energy such as carbohydrates and lipids and adjust their physiology to optimize nutrient uptake (Smith and Yamanaka 2007). In a phosphorus (P)-limited environment, algal cell division is inhibited gradually with the decrease in P, and the growth rate decreased after some days in these new conditions of P availability (Rocha et al. 2016a), highlighting the importance of acclimation to these new environments to better use the available resources (Twiss and Nalewajko 1992, Bonachela et al. 2011, Tantanasarit et al. 2013, Zhang and Hong 2014). Decreases in cell density in all P concentrations were observed, with the growth rate decreasing slightly according to the P supply and being significantly affected just at 2.3 μmol P L^{-1}, which suggests an attempt at keeping the maximum growth rate in exponential phase under suboptimal conditions (Latasa and Berdalet 1994, Geider et al. 1998).

In our study, higher amounts of chlorophyll *a* were obtained under P limitation than in controls, especially at 23 μmol P L^{-1}, differing from the most common pattern seen in the literature which is a decrease in production of this pigment under limiting conditions (Lombardi and Wangersky 1991, Berdalet et al. 1994, Lai et al. 2011, Chia et al. 2013a). This response could be due to the prior acclimation to limiting P, which could be an adjustment of photosynthetic machinery (e.g., electron transport rate) and structure to the available P (Napoleon et al. 2013) as observed in previous studies with P acclimation, where the same pattern was observed (Lombardi and Wangersky 1991). The highest concentration of chlorophyll *a* at 23 μmol P L^{-1} also can be a result of increasing the antenna size and thylakoid stacking, supported by the higher amount of PUFA (Wacker et al. 2015) at this phosphorus concentration.

Biotic or abiotic conditions of cultivation can affect biomass composition (Markou et al. 2012a) and biochemical composition is a result of algal physiological processes and their surrounding environment (Madariaga and Joint 1992; Çelekli et al. 2016). We observed a decrease in dry weight per liter in 23 and 2.3 μmol P L^{-1} that can be a result of fewer cells in the cultures, but per cell, the values are similar in all treatments ($p > 0.05$). These data differ from the literature for another Chlorophyceae (*Ankistrodesmus falcatus*) and one Trebouxiophyceae (*Chlorella vulgaris*), where the increase in weight of P-limited cells was observed (Kilham et al. (1997) and Chia et al. (2013a), respectively), which indicate that the responses for dry weight can be species-specific.

The accumulation of sugars under nutrient stress (Flynn et al. 2010) and their decline under nutrient replete conditions (Turpin 1991) seem to be the most common behavior of algal physiology; however, the response can be species-specific. Nitrogen deprivation results in carbohydrate accumulation in green alga (Thomas et al. 1984) and P deprivation increases the carbohydrates in marine diatoms (Urbani et al. 2005), blue-green alga (Markou et al. 2012b), and in *Chlorella*

vulgaris (Chia et al. 2013b). In our study, the amount of carbohydrates was significantly higher only at the lowest P concentration (≈ twofold higher than control), corroborating previous results observed in *Ankistrodesmus falcatus* (Kilham et al. 1997) grown under P limitation, where the same pattern was observed. This increase can contribute to maintain the cell wall structure (Martínez-Ruiz and Martínez-Jerónimo 2015) and provide energy to deal with stressful situations. The internal cell P concentration available in 2.3 μmol P L^{-1} can be responsible for changes in carbon allocation of *Selenastrum gracile*, as suggested by previous studies, where the carbohydrate synthesis was increased under low internal P (Sigee et al. 2007). Based on our results, we reinforce the importance of previous acclimation to different P availabilities to guarantee that internal P reflects the external availability, especially in short-term experiments.

Under nutrient limitation, a storage of lipids can occur (Lombardi and Wangersky 1991, Wainman et al. 1999) and the higher production is dependent on the effects of nutrient deficiency on growth (Griffiths and Harrison 2009). It is suggested that the cell cycle is inhibited to allow it to accumulate carbon in the form of triacylglycerols (TAG), rich in saturated (SAFA) and monounsaturated (MUFA) fatty acids (Spijkerman and Wacker 2011, Cooksey 2015) that provide the capacity for the cell to handle limitation of a nutrient or excess of a contaminant (Sharma et al. 2012) due to a shift in lipid metabolism (Hu et al. 2008). In our study, a linear increase in lipids per cell with the decrease of phosphorus was obtained, corroborating previous studies (Kilham et al. 1997, Chia et al. 2013a).

Under P limitation, there is an increase in TAG and a decrease in phospholipids (PL) (Lombardi and Wangersky 1991, Chia et al. 2013a), and the TAG to PL ratio usually increases under P limitation (Kilham et al. 1997). It is known that betaine lipids such as diacylglycerol *N*-trimethylhomoserine (DGTS) can increase and replace PL under P limitation (Benning et al. 1995; Khozin-Goldberg and Cohen 2006) and the increase can be due to the exhaustion of nutrients during algal growth (Li et al. 2014). The apparent increase in PL under P limitation observed in our study could be the result of increasing betaine lipid which contains no P and which elutes with polar lipids in the PL band. Since we observed that PL and BL eluted together and we were not able to separate these two lipid classes, we are referring to them as PL + BL. In our study, TAG and PL + BL had almost the same proportion (≈ 37%) in control cells, and these values changed at 23 μmol P L^{-1} (≈ 13% TAG and 59% PL + BL) and at 2.3 μmol P L^{-1} (≈ 23% TAG and 42% PL + BL). As a consequence, the TAG to PL + BL ratio in our experiment decreased to ≈ 1 in control to ≈ 0.21 at 23 μmol P L^{-1}, then increased at 2.3 μmol P L^{-1} (≈ 0.56). Given that TAG is used as storage and PL + BL have structural functions, we suggest that this increase in PL + BL can be an algal attempt to keep its structure under P limitation, as observed when this microalga was exposed to copper and it altered the amounts of ST and PL (Rocha et al. 2016b). In addition, our results suggest that the preferential esterification FA in polar lipids at 23 μmol P L^{-1} was reduced significantly at 2.3 μmol P L^{-1} when growth rate decreased significantly. The lipid class results also differ from others in the literature where FFA was detected only in unstressed cells by some authors (Lombardi and Wangersky 1991, Chia et al. 2013a), while ST can decrease (Kilham et al. 1997, Chia et al. 2013a) or increase (Lynn et al. 2000) under limitation, which agrees with the idea that production and storage of lipids can be species-specific (Shifrin and Chisholm 1981).

Algae can decrease their photosynthetic activity to acclimate to low P (Wu et al. 2012); however, the maintenance of high photosynthetic efficiency can result in higher production of fatty acids (Benvenuti et al. 2015). P limitation changes fatty acid (FA) composition, usually increasing their total amounts, especially in terms of saturated (SAFA) and monounsaturated (MUFA) fatty acids (Goulden et al. 1999, Khozin-Goldberg and Cohen 2006, Spijkerman and Wacker 2011, Chia et al. 2013a), altering membrane structure and fluidity. In our study, we obtained a significant decrease in 18:1ω11 and 20:1ω9, especially at 23 μmol P L^{-1} and 24:0 under limited conditions. A decrease in MUFA was observed under P limitation (at 23 μmol P L^{-1}) and an increase was observed at 2.3 μmol P L^{-1}, which corroborates the literature that shows an increase in this FA group in P-limited environments (Chia et al. 2013a) and the remarkable increase in PUFA at 23 μmol P L^{-1} suggests algal FA reorganization under P limitation. The FA 18:3ω3 increased under P limitation, especially at 23 μmol P L^{-1}.

The increase of PUFA—including the essential fatty acids ARA (20:4ω6) and EPA (20:5ω3)—in P-limited cultures results in a qualitatively rich food for herbivores, albeit in lower quantity, and our results differ from others who obtained a decrease in lower P concentrations (Spijkerman and Wacker 2011, Chia et al. 2013a). The sum of C_{18} essential PUFA (18:3ω3 and 18:2ω6) and sum of ω3 FA also increased with P limitation, especially at 23 μmol P L^{-1} ($p < 0.05$), without differences between control and 2.3 μmol P L^{-1}. The increase of ω3 FA is different from previous results where this sum decreased under P limitation (Chia et al. 2013a). We suggest that the prior acclimation to P altered microalgal physiology and metabolism, e.g., lipid and fatty acid production, and is responsible for the differences observed in our study compared with the literature. One important alteration is the apparent upregulation of desaturases and elongases to increase levels of other members of the ω3 and ω6 PUFA series.

The interpretation of fluorescence parameters is controversial (Kalaji et al. 2014) and previous acclimation to the evaluated conditions can affect the algal response, e.g., some authors state that nutrient-limited algae are more

sensitive to UVB and present photodamage in PSII reaction centers (Bouchard et al. 2008), which would reduce the yield, while others do not observe any relationship between yield and available nutrient to algae (Harrison and Smith 2013). However, the yield values can be less sensitive when the algae were previously acclimated to limited nutrients (Parkhill et al. 2001).

The data obtained using multiwavelength-excitation PAM fluorometry show that photosynthesis of algae changes according to the amount of phosphorus present in the culture medium, and this can be reflected in the maximum and operational yield values, as well as in the nutrient-induced fluorescence transients (NIFT) (Spijkerman et al. 2016), which is the photokinetic response for the resupply of nutrient after limitation (Petrou et al. 2008). Grzesiuk et al. (2016) observed that P internal reserves can be recovered in a short time (less than 2 h) if P-limited algae are enriched with P before being exposed to some pharmaceuticals. The maximum yield provides information about physiological state of phytoplankton while operational yield is related to photosynthetic activity (Kromkamp et al. 2008), with later changes indicating effects on the electron transport chain (Juneau et al. 2002). In our study, less phosphorus in the medium results in lower photosynthetic efficiency, altering the maximum and operational yields only at 2.3 μmol P L^{-1}, indicating that yield (maximum and operational) values were not sensitive parameters in all phosphorus-acclimated algae.

However, looking at quenching obtained with P-acclimated algae at 72 h, it is possible to infer that non-photochemical quenching (Stern-Volmer coefficient-NPQ- and qN), which can be used as a stress indicator (Misra et al. 2012), was more sensitive to phosphorus limitation than yield values, with significant differences in the treatments compared to control. Changes in non-photochemical quenching are related to an increase in heat energy dissipation (Ralph and Gademann 2005), which can be caused by changes in pH across the thylakoid membrane (Wykoff et al. 1998) and is a photoprotection mechanism in nutrient-stressed cells (Petrou et al. 2008).

The cell changes we observed under P limitation suggest that the microalga *S. gracile* responds to phosphorus limitation, by adjusting growth, photosynthesis, and chlorophyll synthesis and increasing carbohydrate and lipid and fatty acid production, in an attempt to maintain physiological and biochemical processes at the highest rate possible. This is especially true at 23 μmol P L^{-1}, where several maximum results in specific fatty acids, e.g., sum of C_{18} PUFA essential fatty acids, 18:3ω3, 18:4ω3, sum of ω3 fatty acids and PUFA, and chlorophyll *a* were observed. However, the non-linearity of responses from control to the lowest concentration (2.3 μmol P L^{-1}) indicates that after some threshold, it seems that algae are unable to continue using their machinery at a maximum to avoid damage. Between the control (230 μmol P L^{-1}) and 23 μmol P L^{-1}, there was an increase in lipids, which largely consisted of PUFA including HUFA (highly unsaturated fatty acids) going into membranes, which explains the increase in polar lipid values, while between 23 and 2.3 μmol P L^{-1}, there was a reorganization with HUFA and MUFA replacing PUFA, which could be the responsible for the changes in photosynthesis. Since the unsaturation of FA can affect the photosystem II tolerance to environmental stress, we suggest that the highest amount of PUFA at 23 μmol P L^{-1} plays an important role in membrane fluidity, the electron transport rate, and the maximum growth rate, as well as increasing the chlorophyll production, indicating that algal metabolism is at its maximum, even with limitation of P. However, at lower P concentrations, this alga is unable to maintain its metabolism and this reflects the highest non-photochemical values, probably due to photoinhibition—which is linked to the highest MUFA value and lowest growth rate. Based on the different results obtained in our study, prior acclimation to P-limited media affected microalgal physiology and metabolism altering responses, and the changes in fatty acids as a result of P availability led the changes in growth and photosynthesis.

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