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Intraspecific variation in alkaline phosphatase activity in *Phaeodactylum tricornutum* (Bacillariophyceae, Bohlin)

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ABSTRACT

To describe potential intraspecific variation in phosphorus incorporation in two strains of *Phaeodactylum tricornutum* (Bohlin), Ub3 and Ub7, alkaline phosphatase (AP) activity was evaluated via enzyme-labeled fluorescence assay. Analysis using the probe ELF-97® provides individual evaluation, and therefore can determine the nutritional status of inorganic phosphorus in phytoplanktonic cells. Bioassays compared the control treatment to both phosphate-enriched and phosphate-depleted treatments by varying only the phosphate concentration in the media. The *P. tricornutum* strains exhibited differences in their development when incubated in the phosphate-enriched media. The development of the Ub7 strain differed by exhibiting "luxury uptake" and utilization of organic phosphorus, and the alkaline phosphatase analysis indicated limitations of this clone under such conditions. The Ub7 strain showed higher AP activity, when compared to Ub3, in the P-enriched condition. *P. tricornutum* presented increases in AP activity and low variation in Surface/Volume ratio, by increasing biovolume and its maximum linear dimension, as strategies for phosphate incorporation. Our results highlight intraspecific differences in alkaline phosphatase activity, and hence differences in the incorporation of organic phosphorus, as the tested species regulated enzymatic activity under different external phosphate concentrations.

Keywords: alkaline phosphatase, auxotrophy, marine diatom, organic phosphorus incorporation, phosphate limitation

Introduction

Biochemical characteristics of microalgae are dependent on taxonomy as well as on individual physiological characteristics and their response to light and nutrient availability. For example, the same concentration of a nutrient could mean repletion for one species and nutritional stress for another. Internal concentrations of proteins, nucleic acids, phospholipids, phosphorus reserves and nitrogen depend on cell nutritional history and physiological and taxonomic differences (Falkowski 2000; Vance *et al*. 2003). One way to assess differences in nutrient incorporation within and among species is by studying the activity of a specific enzyme.

Phosphorus (P) is a limiting nutrient in some freshwater and coastal marine ecosystems (Gregoracci *et al*. 2012; Maitra *et al*. 2015). According to Strojsová *et al*. (2003) and Reynolds (2006), the enzymatic production of phosphatases, which hydrolyze dissolved organic compounds, is an important physiological trait that allows some species to endure conditions of limited phosphate. Alkaline phosphatase (AP) is an extracellular enzyme expressed by

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a wide variety of phytoplankton species in response to the condition of limited phosphorus. Highly stable in seawater, it is a membrane-associated enzyme that catalyzes the hydrolysis of phosphorus-containing organic compounds such as phosphate esters (Fitzgerald & Nelson 1966; Graziano *et al*. 1996; Dyhrman 2005; Pandey & Parveen 2011), to obtain orthophosphate, a form of inorganic phosphorus directly available to organisms (Pandey & Parveen 2011). In other words, alkaline phosphatase facilitates the use of dissolved organic phosphorus (DOP) by phytoplankton when dissolved inorganic phosphorus (DIP) is limited in the environment (Lin *et al*. 2011). The activity of alkaline phosphatase is related to different phosphorus concentrations (Hernández *et al*. 2002), with it being produced when cells are experiencing phosphate deprivation or it being suppressed in conditions of sufficient phosphate (Kuenzler & Perras 1965; Fitzgerald & Nelson 1966; Grainger 1989; Vance *et al*. 2003).

Many phytoplankton species have increased alkaline phosphatase (AP) activity in P-limited environments, and so its activity can be used as an indicator of phosphorus deficiency in these organisms (Rengefors *et al*. 2001). Previous studies have observed an increase in AP activity under conditions of depletion of inorganic P in *Chlorella pyrenoidosa*, *Scenedesmus dimorpha*, *Microcystis aeruginosa* and *Aphanizomenon flos-aquae* (Beardall *et al*. 2001); *Alexandrium fundyense*, *Amphidinium* sp. and *Isochrysis galbana* (González-Gil *et al*. 1998); and *Phaeodactylum tricornutum* (i.e. Lin *et al*. 2013; Yang *et al*. 2014). On the other hand, Marco & Orus (1988) observed that the cyanobacteria *Trichodesmium* sp. and *Anabaena* sp., under similar conditions of P limitation, showed no significant increase in the production of AP. These findings demonstrate that each species responds differently to the different environmental conditions of either sufficient or depleted nutrients (Rengefors *et al*. 2001).

This study evaluates the incorporation of phosphorus in two clones of *Phaeodactylum tricornutum* in order to describe that allow these organisms to out-compete other individuals in conditions of phosphate deprivation, and to be outcompeted in conditions of replete phosphate.

Materials and methods

Sterilization and pre-experimental preparation

Glass vessels were cleaned by immersing them in a 10% chloridric acid bath for 24 hours, rinsed with abundant distilled water, dried in an area protected from dust and autoclaved (30 minutes, 121° C), according to the protocols described in Kawachi & Noel (2005). Plastic vessels that could not be autoclaved were cleaned as described above and then sterilized under UV radiation (260 nm) for 10 minutes (Kawachi & Noel 2005).

The seawater used in all experimental treatments and as a control was collected with a Van Dorn bottle from coastal waters (southeastern Brazil near Ilha Grande- RJ), filtered through a 150-µm mesh to remove microzooplankton and through 0.45 and 0.22 µm cartridges (Milipore®), to eliminate phytoplankton and bacterioplankton. The water was then sterilized by UV radiation and autoclaved prior to use in the experiments.

Organism and culture conditions

The unicellular diatom *Phaeodactylum tricornutum* (Bacillariophyceae; strains Ub3 and Ub7 available at the Instituto Oceanográfico, Universidade de São Paulo Marine Microorganisms Culture Collection) used in this study was isolated from coastal waters in southeastern Brazil (Ubatuba, state of São Paulo). These non-axenic strains have been maintained in the Marine Microalgae Culture Collection of the Faculdade de Oceanografia, Universidade do Estado do Rio de Janeiro in Guillard F/2 medium (Guillard & Ryther 1962) for many generations. Prior to experimentation, cells were batch-cultured in 500 mL Erlenmeyer flasks containing 250 mL of enriched seawater (34 salinity) with F/2 medium until the end of the log growth phase (5-8 days). The cells were then washed by centrifugation (three times, 2000 x g for 10 minutes) in control seawater and remained for 12 hours in 500 ml of this water for starvation. During the experiments, cultures were exposed to 80 $\mu E.m^{-2}.s^{-1}$ PAR provided by fluorescent lamps (Sylvania cool white tubes, 40 W), under a 12:12 h light:dark cycle and a temperature of 20° C (± 0.5° C). Cultures were manually shaken two times a day. Cell growth was followed by direct microscopic cell counts of culture subsamples, using a *Sedgewick Rafter Counting Cell,* after treating with acidic Lugol's solution, and by optical density. Cultures were set at an initial cell density of about 1.0 x 10^3 $cells.mL^{-1}$ and inoculated with three treatments by the addition of silica: 1) Control - control water enriched with Guillard F/2 medium (36 µM); 2) F (Phosphate-enriched) - treatment with F/2 enriched with phosphate at medium proportion of F (72 µM); and 3) F/8 (Phosphate-depleted) - treatment with F/2 under phosphate deprivation at medium proportion of F/8 (4.5 µM) (Guillard & Ryther 1962). Bioassays were performed in triplicate within 15 days, and cell number was used to calculate the growth rate of all treatments.

Alkaline phosphatase incorporation assays

Alkaline phosphatase activity of the tested microalgae was determined using an ELF-97 endogenous phosphatase detection kit (Molecular Probes, E-6601®) following the method of Gonzalez-Gil *et al*. (1998), as modified by Dyhrman & Palenik (2001) and Skelton *et al*. (2006). Aliquots of 5 mL of each culture were removed during late exponential (12 day culture) and stationary (15 day culture)

growth phases, fixed with 1 mL of 70% ethanol for 30 minutes and washed by centrifugation at 2000 x g for 10 minutes. The supernatant was then aspirated with the aid of a vacum pump.

Prior to use, the ELF-97® substrate was diluted in ELF detection buffer at a ratio of 1:20 and filtered through a 0.2 µm cartridge (Milipore®); 100 µL of this solution was added to each sample, which were then incubated at 20°C for 30 minutes. Following incubation, the cells were washed and centrifuged (2000 x g for 10 minutes) in 1 mL solution of 10 mM phosphate buffered saline (PBS) for five times, resuspended in 1 mL PBS and filtered (3 µm black membranes- Poretics®). Slides of the resulting product were prepared for observation under an epifluorescence microscope.

The microalgae were analyzed to determine the enzymatic activity of alkaline phosphatase (fluorescence of ELF-97®) using a Nikon® epifluorescence microscope (model Eclipse E200), equipped with a mercury lamp of 100W and an epifluorescence filter device with excitation at 360 ± 40 nm and emission at 535 ± 50 nm (# 31060v2; Chroma Technology Corp., Rockingham, VT).

Qualitative analysis involved counts of total cells and cells with ELFA precipitate (termed labeled cells). Images were obtained with the aid of a digital camera (Canon®) attached to the epifluorescence microscope.

Cell volume

Cell volume and surface area were estimated at the end of the experiment (stationary phase) by measuring 40 cells randomly chosen from each clone and each treatment, according to the formulation proposed by Sun & Liu (2003), using an inverted optical microscope.

Phosphate analysis

The phosphate concentration of each treatment was analyzed during the beginning of the experiment (time zero), and during late exponential (12 day culture) and stationary (15 day culture) growth phases, using FIA (Flow Injection Analysis – FOSS Tecator FiaStar 5000 analyzer—FOSS analytical, Hillerod, Denmark). Analyses were performed according to the specifications and recommendations of Note AP 5201 (ISO 13395: 1996-revision 2, detection limit 0.05 µmol.L^{-1}).

Functional traits

Half-saturation constant (Ks – µM)

The clones of *P. tricornutum* were grown in continuous cultures (1L) in F/2 medium (Guillard & Ryther 1962). The experiment was repeated three times, and the data were considered as triplicates. Cells (10^6 cells.L^{-1}) were maintained in suspension using a magnetic stirrer at 30 rpm in the culture conditions described in item 2.2. The population reached the steady state between 10 and 12 days, when cell concentration varied less than 5 %. Until reaching the steady state, cell density data was used to calculate growth rates.

Upon reaching steady state, 10 µM of phosphate (final concentration) was added to the continuous culture. After this pulse, 5 mL subsamples were taken every 15 minutes for 10 hours (time experimentally determined by Riegman *et al*. 2000), and filtered through a 0.2 µm membrane (Acrodisc, Gelman Sciences®). This experiment was designed to determine the rate of cellular uptake (Vt) at a given time (t) by the maximum rate of incorporation established immediately after the pulse of 10 µM phosphate:

$$V_t = V_{max}\left(1 - \frac{\Delta Q}{Q_{max\,t}}\right)$$

Where V_t (which is equal to $^{(x-1)} \times \left(\frac{dS}{dt}\right)$; where is the cell concentration and S is the external nutrient concentration) is the cellular uptake rate at time t. V_{max} is the maximum incorporation rate measured at t = 0; ΔQ is the amount of phosphate incorporated at time t, and $Q_{max\,t}$ is the maximum amount of phosphate that cells can incorporate after the pulse at a given time t. The half saturation constant (ks) was estimated by ks = $\frac{1}{2} \times V_{max}$ (Riegman & Mur 1986).

Cell quota (Q – µM)

The phosphate ate was obtained by subtracting the final concentration of phosphorus from its initial concentration in the medium during the stationary phase, since this latter phase is with nutrient limitation and exhibited maximum cell growth.

Intracellular phosphorus ([P] int. - pg P.$Cell^{-1}$):

Intracellular phosphorus was calculated by normalizing the cell quota per total cellular density in the steady state.

Statistical analysis

The results were analyzed by a Nonparametric Kruskal-Wallis ANOVA and Median Test, followed by Multiple comparisons of mean ranks for all groups, with a significance level of α = 0.05, using Statsoft Statistica 7.0 software.

Results and discussion

The growth of the *Phaeodactylum tricornutum* strains Ub3 and Ub7 under laboratory conditions showed a lag phase of 4 days. After this time, cells grew actively up to the stationary phase with the Ub3 clone having higher growth rates in all treatments than the Ub7 strain (Tab. 1), with the exception of the F/8 treatment in which both grew at

Table 1. Growth rate (μ - d^{-1}) and nutritional condition of *Phaeodactylum tricornutum* strains Ub3 and Ub7 cultured under Phosphate-enriched (Control and F treatment) and Phosphate-depleted (treatment F/8) conditions.

Strains	μ (d^{-1})	Nutritional Condition
Ub3	0.97	P-enriched (C - 36 μM)
	0.98	P- enriched (F - 72 μM)
	0.69	P- depleted (F/8 – 4.5 μM)
Ub7	0.78	P- enriched (C - 36 μM)
	0.78	P- enriched (F - 72 μM)
	0.56	P- depleted (F/8 – 4.5 μM)

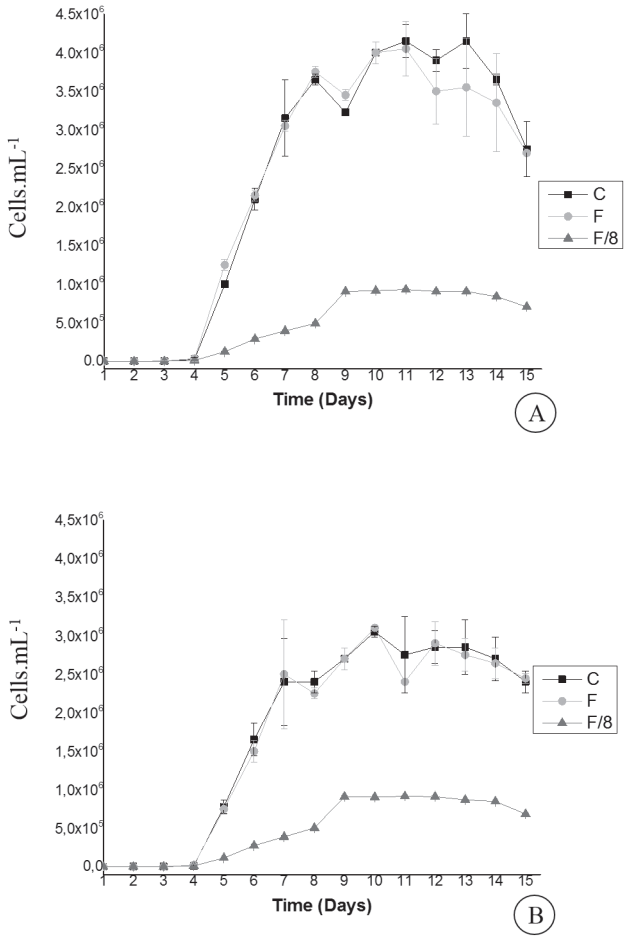

Figure 1. Growth of *Phaeodactylum tricornutum* strains Ub3 (A) and Ub7 (B) in Control (C), F and F/8 treatments.

similar rates and achieved similar densities (Fig. 1A-B).

The highest densities of Ub3 were observed at the end of the exponential growth phase (10-13 days) in the control ($4.1 \times 10^6 \pm 2.1 \times 10^5$ cells.mL^{-1}) and the F ($4.0 \times 10^6 \pm 1.4 \times 10^5$ cells.mL^{-1}) treatment (Fig. 1A). During the same period, the densities of Ub7 in the F treatment and the control were similar ($3.0 \times 10^6 \pm 7.0 \times 10^4$ cells.mL^{-1}) (Fig. 1B). Although phytoplankton adjust their growth rates to changes in environmental conditions, variation in maximum cell densities may be explained by differences in experimental conditions, such as the phosphate concentration of the culture media (Harris 1978).

In the literature, *P. tricornutum* is frequently reported as an opportunistic species with fast growth (Falciatore *et al*. 1999; Martino *et al*. 2007; Desbois *et al*. 2010), as was observed in the present study (see growth curves in Fig. 1A-B). This species reaches the stationary phase from the forth to the seventh day of growth, with maximum cell density varying from 10^4 to 10^7 cells.mL^{-1}. As with growth rate, maximum cell density can vary considerably among strains of the same species under the same culture conditions (i.e. Aidar *et al*. 1991; Voltolina *et al*. 1998; Foster *et al*. 2008; Ohse *et al*. 2008; Lin *et al*. 2013).

Both strains of *P. tricornutum* had lower growth rates when subjected to P-depleted conditions. Specific growth rates observed in the tested strains were similar to those described by Gaeta (1985), who reported growth rates of 1.13 ± 0.06 d^{-1} and 1.39 ± 0.08 d^{-1} in phosphate-enriched media (105 μM and 210 μM), and by Lin *et al*. (2013), who observed rates of 0.8 d^{-1}in non-limited P-conditions.

Concerning biovolume, the Ub3 strain presented a lower biovolume and a surface/volume ratio (S/V) similar to that of the Ub7 strain, as was expected for an organism developing in a P-depleted medium (Tab. 2). However, the highest changes were observed in the Ub7 strain, with maximum biovolume in the P-depleted treatment, despite having a S/V ratio similar to the control (Tab. 2). This variation among treatments is probably related to the growth phase, since measurements were performed in the stationary phase with the cells limited by nutrients (Timmermans & Wagt 2010). The two strains of *P. tricornutum* adapted to low phosphorus concentrations by increasing, as observed in Ub7 strain, or decreasing, as employed by Ub3 strain, biovolume, and slightly altering S/V ratios. Thus, the Ub7 strain could better exploit the absorption of this nutrient in the depleted treatment since increased biovolume and maintenance of S/V ratio are related to absorption and intracellular phosphorus storage (*luxury uptake*).

The activity of alkaline phosphatase in the two strains of *P. tricornutum* exhibited different responses for incorporating phosphorus into their cells under different conditions of phosphate availability. The Ub3 strain exhibited higher enzymatic activity in the P-limited treatment (Fig. 2A-B), although the difference was statistically significant only during the stationary growth phase ($p < 0.05$; Tab. 3), indicating that AP activity is higher in cells under conditions of low phosphate concentrations. These results agree with previous studies (González-Gil *et al*. 1998; Beardall *et al*. 2001; Dyhrman & Palenik 2001; Moser *et al*. 2010; Yang *et al*. 2014), since this microalgae is able to use a strategy of organic phosphorus incorporation when the inorganic form of this nutrient is limited (Rengefors *et al*. 2001; Nedoma *et al*. 2003). On the other hand, the control and F treatment

(P-replete conditions) for Ub7 exhibited greater proportions of labeled cells in the exponential growth phase compared to the P-limited treatment (Fig. 3A), results also found for diatoms of a freshwater reservoir and hypereutrophic lakes (Strojsová *et al*. 2003; Cao *et al*. 2005). However, despite the increase in labeled cells, these treatments did not differ significantly from each other ($p > 0.05$; Tab. 3), indicating phosphate limitation for the Ub7 strain in all tested conditions. At stationary growth phase, the enzymatic activity was similar to that observed for the Ub3 strain, with a greater number of labeled cells being found in the P-depleted treatment ($p < 0.05$; Tab. 3). It is noteworthy that the phosphate cell quota in the Ub7 strain, in both treatments, was lower than those of the Ub3 strain (Tab. 4), which suggests increased uptake of phosphate by this algae.

Intraspecific variation in AP activity in *P. tricornutum* has been previously reported, hence this microalgae is used worldwide in bioassays of algal growth potential (Yang *et al*. 2014 and references therein). Lin *et al*. (2013) observed that *P. tricornutum* growing in a starved medium had intense AP activity from the fourth to the seventh day of incubation, and that AP mRNA expression was abundant. Additionally, Yang *et al*. (2014) found two genes responsible for encoding alkaline phosphatases in *P. tricornutum*, which exhibit sharp rises in their transcription when limited by phosphorus. In such conditions, the increase in AP activity may be related to the incorporation of organic phosphorus. In contrast, Ruiz *et al*. (1997) found that *P. tricornutum* growing in phosphate concentrations ranging from 2.5 to 50 µM, exhibited no significant differences in alkaline phosphatase activity. However, when the microalgae were incubated at concentrations higher than 50 µM, a large decrease in enzymatic activity was observed after four days until it ceased its production.

The activity of AP seems to be controlled by the availability of inorganic phosphorus for some, but not all, phytoplankton species, suggesting that enzyme activity is not a universal measure of phosphorus requirement (Rengefors *et al*. 2001). In the experiments conducted by Marco & Orus (1988), the cyanobacteria *Trichodesmium* sp. and *Anabaena* sp. exhibited no significant increase in AP in limited concentrations of inorganic phosphorus, while Keenan & Auer (1974), detected no AP activity in *Microcystis aeruginosa* and *Selenastrum capricornutum*. Experiments conducted using P-starved *Microcystis aeruginosa* cells indicated that colonial morphotypes grow in low P levels, while unicellular morphotypes consumed more P and had higher alkaline phosphatase activity in P-depleted conditions (Shen & Song 2007).

An inverse relationship between the intracellular concentration of phosphorus, cell quota (Q) and the phosphate content of the medium is well established (Kuenzler & Ketchum 1962; Fitzgerald & Nelson 1966; Hernández *et al*. 1993; Ruiz *et al*. 1997). Kuenzler & Ketchum (1962), found that cells of *P. tricornutum* growing in high concentrations of phosphate (80 µM) exhibited a high cell quota after a long period of nutrient accumulation, while cells incubated under limiting conditions were capable of removing almost all of the nutrients from the media before significant cell multiplication occurred. As culture growth continues, the intracellular concentration of phosphorus declined in the limited treatment (8 µM) indicating that *P.*

Table 2. Cell volume (µm³), maximum linear dimension (MLD) and surface/volume ratio (S/V) *Phaeodactylum tricornutum* strains Ub3 and Ub7 in Control (C), F and F/8 treatments, at stationary growth phase.

P. tricornutum		Biovolume	MLD	S/V
		µm³	µm	µm⁻¹
Ub3	Control	62.09	6.79	2.01
	F	61.63	5.43	1.74
	F/8	48.81	6.04	2.21
Ub7	Control	65.30	6.24	1.81
	F	72.17	4.79	2.34
	F/8	85.20	4.95	1.90

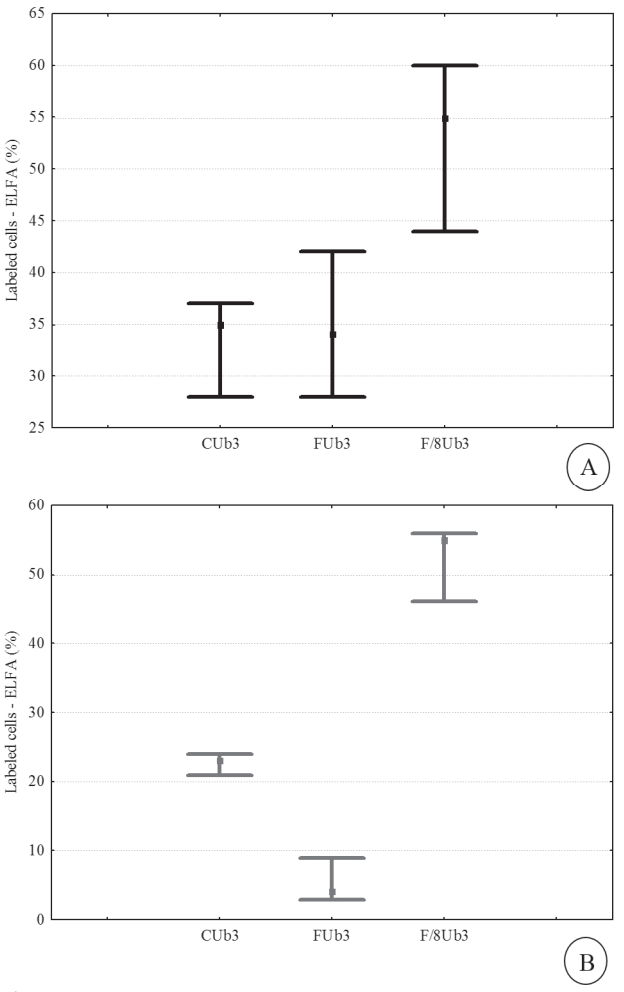

Figure 2. Percentage of labeled cells (ELFA) of *Phaeodactylum tricornutum* strain Ub3 in Control (C), F and F/8 treatments, at late exponential (A) and stationary (B) growth phases; (■) Median; ( ) 25% - 75%.

Table 3. Kruskal-Wallis ANOVA test (H) followed by Multiple Comparisons z' test of *Phaeodactylum tricornutum* Ub3 and Ub7 strains in control (C), F and F/8 treatments; significant differences highlighted; confidence interval of 95%.

Kurskal-Wallis ANOVA (H)				
	Exponential Phase		Stationary Phase	
	H	p	H	p
Ub3	5.42	0.06	**7.26**	**0.02**
Ub7	4.26	0.11	**6.48**	**0.03**
z' Test - Multiple Comparisons				
	Exponential Phase		Stationary Phase	
	p		p	
CUb3 X FUb3	1.00		0.53	
CUb3 X F/8Ub3	0.15		0.53	
CUb3 X CUb7	**0.03**		1.00	
CUb3 X FUb7	0.43		1.00	
CUb3 X F/8Ub7	1.00		0.83	
FUb3 X F/8Ub3	0.11		**0.02**	
FUb3 X CUb7	**0.04**		0.99	
FUb3 X FUb7	0.53		1.00	
FUb3 X F/8Ub7	1.00		**0.05**	
F/8Ub3 X CUb7	1.00		1.00	
F/8Ub3 X FUb7	1.00		0.70	
F/8Ub3 X F/8Ub7	1.00		1.00	
CUb7 X FUb7	0.22		0.89	
CUb7 X F/8Ub7	0.22		0.40	
FUb7 X F/8Ub7	1.00		**0.03**	

tricornutum had become phosphorus deficient and stopped multiplying.

The intracellular concentration of phosphorus of the Ub7 strain observed in this study ranged from 0.6 to 4.7 pg P. Cell^{-1} in P-depleted (F/8) and P-replete (F) treatments, respectively (Tab. 4). Nevertheless, the control for both strains showed intracellular P concentrations (~2.0 pg P. Cell^{-1}) similar to those reported by Kuenzler & Ketchum (1962) when phosphate depletion limited cell growth (8µM). It should also be noted that in the present work, the highest cell quota and intracellular concentration of phosphorus were observed in the Ub7 strain in the P-replete condition (Tab. 4), suggesting that this strain possesses a large capacity to store phosphorus, which has also been described for *P. tricornutum* by Terry *et al*. (1983) and diatoms and other organisms of a natural community (Domingues *et al*. 2011; 2015).

The two strains of *P. tricornutum* exhibited different phosphate half-saturation constants (Ks). The Ks value estimated for Ub7 was twice that of Ub3, indicating lower affinity for phosphate, which can be further enhanced throughout growth. These results indicate that under P-enriched conditions (72 µM) the Ub7 strain of *P. tricornutum* has a greater ability to store intracellular phosphorous. However, this phosphate pool is not incorporated into the maintenance of cellular metabolism in the same proportion that it is in Ub3, since lower cell densities were observed in all treatments (Fig. 1B).

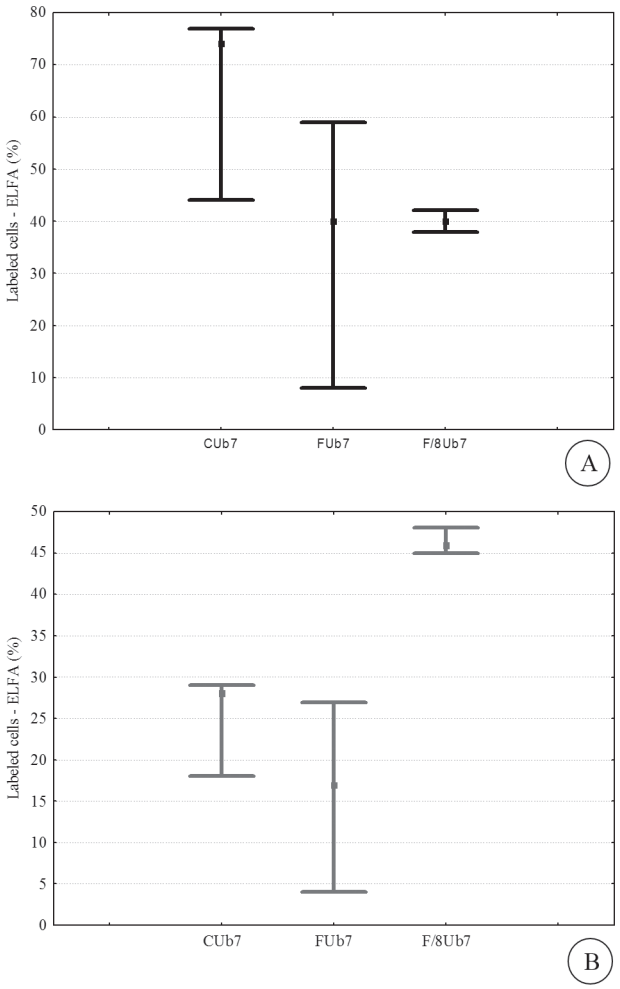

Figure 3. Percentage of labeled cells (ELFA) of *Phaeodactylum tricornutum* strain Ub7 in Control (C), F and F/8 treatments, at late exponential (A) and stationary (B) growth phases; (■) Median; ( ) 25% - 75%.

According to Monod (1949), higher Ks values are correlated with a lower affinity for a specific nutrient by microorganisms, as well as lower specific growth rate. Thus, when nutrient concentration is low in a culture medium, the growth rate becomes limited and dependent on this nutrient. Different competitive strategies for phosphate are possible (Sommer 1984). One possibility is that species with smaller Ks values have greater affinity for absorption of nutrients providing an advantage in nutrient limited environments. In this sense, the lowest value of Ks and higher cell densities observed in Ub3 suggest a strategy to more effectively exploit the availability of phosphate, especially in the limited treatment. On the other hand, species with high nutrient absorption and lower growth rates favor nutrient storage inside the cells. This excessive consumption ("luxury uptake"), which refers to the absorption and storage of phosphorous at levels greater than those required for immediate growth, could favor these species during periods of reduced availability of nutrients (Keenan & Auer 1974; Mackey *et al*. 2012; Wasmund *et al*.

Table 4. Half-saturation constant (Ks – μM), cell quota (Q – μM), intracellular phosphorus ([P] int. - pg P.Cell^{-1}), phosphate concentration in the medium ([P] Treatment - μM) and nutritional condition (μM) of *Phaeodactylum tricornutum* strains Ub3 and Ub7 cultured under Phosphate-enriched (treatments control and F) and Phosphate-depleted (treatment F/8) conditions; *At stationary growth phase.

Phaeodactylum tricornutum					
	Ks (μM)	**Q (μM)**	**[P] int. (pg P.cell^{-1})**	**[P] Treatment (μM)**	**Nutritional Condition**
Ub3	0.68	21.3	1.6	14.9*	P-enriched (C - 36 μM)
		31.4	2.2	41*	P- enriched (F - 72 μM)
		1.8	0.5	2.7*	P- depleted (F/8 – 4.5 μM)
Ub7	1.58	26.1	2.0	10.15*	P-enriched (C - 36 μM)
		61.4	4.7	11*	P- enriched (F - 72 μM)
		2.1	0.6	2.4*	P- depleted (F/8 – 4.5 μM)

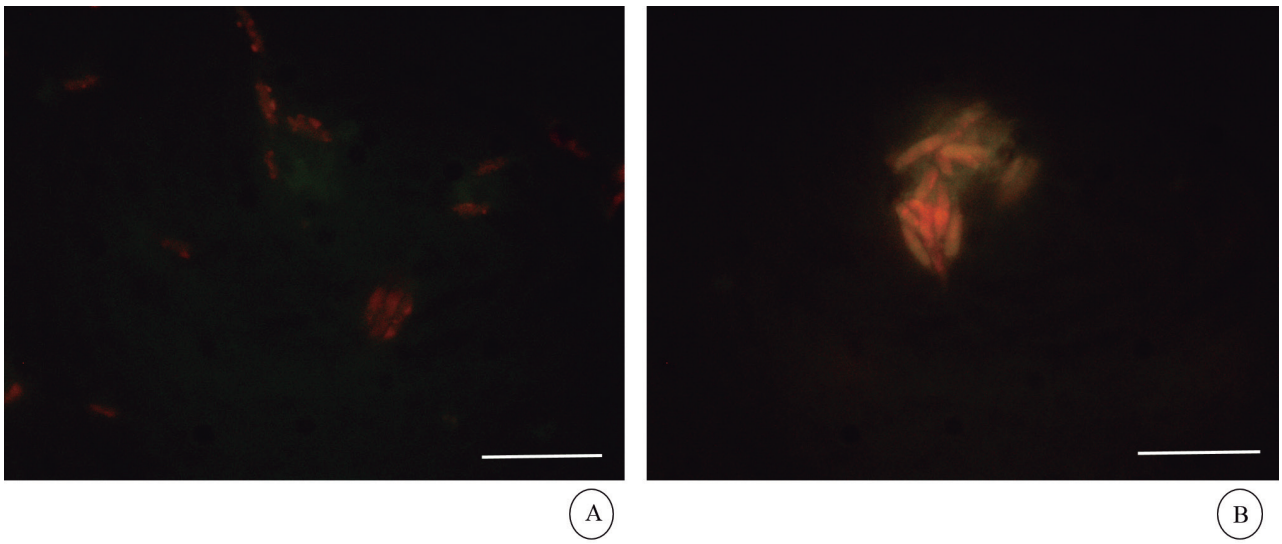

Figure 4. *Phaeodactylum tricornutum* strain Ub7, at stationary growth phase (epifluorescence microscopy; 1000x). Cells without alkaline phosphatase activity, cultured under treatment control (A) and with alkaline phosphatase activity, cultured under Phosphate-depleted (treatment F/8) conditions (B). Scale 50 μm.

2014; Domingues *et al*. 2015). This particular uptake was shown by the Ub7 clone and evidenced by it having the highest half-saturation constant and higher intracellular concentrations of phosphorus in the P-enriched treatment. Consequently, cell division may proceed for a certain period, without subsequent nutrient uptake (Kuenzler & Ketchum 1962), since phosphate reserves can sustain growth for several generations in the absence of phosphorus in the medium (Fitzgerald & Nelson 1966). The prompt absorption of nutrients is a common strategy by which phytoplankton exploit the nutrient resources available in oligotrophic waters (Goldman & Glibert 1982), while the absorption rate may increase up to 100 times in cells limited by phosphorus (Brown *et al*. 1978).

In accordance with Aidar *et al*. (1991), the highest phosphate absorption rates occurred during the lag phase of cell growth, indicating that phosphate absorption occurred immediately. However, significant increase in biomass only occurred late in the exponential phase. These results demonstrate that the processes of absorption and assimilation are different, and their indicator rates do not occur simultaneously (Goldman & Glibert 1983; Collos 1986). For many microalgae, the processing of absorbing nutrient metabolism into biomass is not instantaneous and requires a particular period of time that varies among species. For some organisms, the lag phase of cell division is so extensive that absorption and growth are not on the same time scale. These different scales have important implications for interpreting competition among species for limiting nutrients (Collos 1986).

Experimental results show that cells in conditions of phosphorus deficiency can achieve higher absorption rates than cells in conditions of phosphate saturation. In those deficiency circumstances, *P. tricornutum* are able to absorb from 8 to 16 times as much as the minimum cell quota, by converting phosphate into intracellular polyphosphate reserves to support three to four generations in conditions of phosphate depletion (Yao *et al*. 2011). The isolated strains of the same species may reflect significant genetic variability that is reflected in variability of characters of cellular physiology (Gallagher 1980; 1982; Huang *et al*. 2011; Yang *et al*. 2014). This agrees with our results, since Ub3 and Ub7 both showed different responses in relation to absorption and phosphate assimilation, as shown by

their absorption rates (Tab. 4).

Additionally, Yang *et al*. (2014) analyzed transcriptional changes in *P. tricornutum* under phosphorus stress and found two genes encoding phosphatases, and 11 encoding phospholipases involved in certain key genes for adaptation to P limitation. The sequencing of the same species by Huang *et al*. (2011), revealed 13 novel microRNAs (miRNAs) that perform specific regulatory functions in metabolism related to the synthesis of fatty acids and the urea cycle under nitrogen and silica limitation. When restricted to low iron concentrations, *P. tricornutum* employed metabolic rearrangements to acclimatize to the restricted nutrient levels (Allen *et al*. 2008). According to Terry *et al*. (1983), the physiological differences between strains of *P. tricornutum* may be greater than those found among different species of microalgae. In Gaeta (1985), statistical differences found in physiological parameters, such as growth rate, productivity and carbon assimilation, and the polymorphism shown by the clones, endorse the existence of different physiological strains of the same species (*P. tricornutum*). Furthermore, Martino *et al*. (2007) analyzed intraspecific genetic diversity of 10 strains and found indications of four different genotypes that exhibited distinct phenotypic characteristics related to the physiological differences of the morphotypes of *P. tricornutum*.

Many species of microalgae are able to obtain phosphorus from phosphate esters to sustain growth in an environment restricted by orthophosphate, and different algae differ in the presence and location of phosphatases (González-Gil *et al*. 1998; Dyhrman & Palenik 1999; Rengefors *et al*. 2001). The incorporation of organic nutrients by *P. tricornutum* is widely known, with alkaline phosphatase activity consistently bound to the cells (Kuenzler & Perras 1965) and the plasma membrane as the site of greater specificity of PA (Flynn *et al*. 1986). Likewise, in this study, the greatest ELFA precipitation was observed around the cells of *P. tricornutum* and attached to the plasma membrane (Fig. 4B).

Conclusions

Despite *Phaeodactylum tricornutum* usually having greater enzymatic activity in conditions of phosphate depletion, intraspecific variation was observed.

The Ub7 strain displays deficiency in phosphate incorporation, even in P-enriched conditions when there was excessive consumption of phosphate, alkaline phosphatase activity and use of P-organic. Through the use of organic phosphorus, *P. tricornutum,* in exponential growth phase, is capable of storing a higher internal concentration of phosphorus, however, this storage does not reflect an increase of cell density in the stationary phase, showing a probable imbalance between the absorption and assimilation of this nutrient.

The differences observed between the strains may be the result of a higher capacity for absorbing the nutrient by *P. tricornutum* Ub7, specified by the excessive consumption, or to different physiological conditions of the cells that interfere with their metabolic and chemical composition relations. Perhaps the incorporation of organic phosphorus and the modification of cell volume and length are mechanisms used by Ub7 to increase the orthophosphate cell quota, even in P-enriched conditions.

Batch cultures undergo continual changes in their chemical composition, especially with regard to nutrient concentration, which are reflected in metabolism and cellular biochemistry and, consequently, changes in surface/volume ratio and other physiological interactions that occur during different growth stages of a microalgae.

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