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Changes in motility and induction of enzymatic activity by nitrogen and phosphate deficiency in benthic *Halamphora luciae* (Bacillariophyceae) from Argentina

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Abstract: Cells of *Halamphora luciae* were isolated from the mudflat of the Bahía Blanca estuary. Unialgal axenic cultures were grown in f/2 with limiting concentrations of N or P and in f/2 medium deprived of one or both macronutrients. On the one hand, limited macronutrient supply did not affect growth rate, but final cell yield was lower than in the complete medium. On the other hand, N or P deprivation reduced growth rates as well as final cell yield in the stationary phase. P deficiency enhanced the secretion of soluble extracellular polymeric substances (EPS), the percentage and speed of gliding cells, as well as the activity of alkaline phosphatase, especially after a previous prolonged P starvation. Regarding the morphology of the frustules, P deprivation reduced areola size but did not alter cell biovolume. N deficiency had a deleterious effect on the motility of the cells, and reduced the secretion of soluble EPS and cell biovolume. After incubation in N-deprived media for 10 days, cells exhibited enhanced protease activity.

Key Words: Alkaline phosphatase, Biovolume, EPS, Frustule morphology, *Halamphora,* Motility, Nitrogen and phosphorus deficiency, Protease

INTRODUCTION

Intertidal mudflats are highly dynamic systems subjected to rapid changes in environmental variables such as salinity, irradiance, temperature, etc. Despite these unstable conditions, intertidal mudflats are highly productive systems where epipelic diatoms are frequently the main primary producers (Brandini *et al*. 2001; Popovich & Marcovecchio 2008). Epipelic diatoms form biofilms by secreting a considerable part of the photosynthetically fixed carbon (Myklestad *et al*. 1989) as extracellular polymeric substances (EPS) that embed the cells in an abundant mucilaginous secretion attached to the sediments. Here they coinhabit with other microbenthic organisms and participate in diverse biological and biogeochemical processes (Decho 2000; Underwood & Paterson 2003).

Movement in epipelic diatoms is coupled to mucilage secretion along the raphe or through the apical pore fields in araphid diatoms (Molino & Wetherbee 2008). Force generation for such movements requires a motor protein operating on underlying actin filaments (Cohn *et al*. 2003; Molino & Wetherbee 2008 and literature within). EPS secretion provides not only movement and hence the ability for the cells to respond to the environment, but it also leads to sediment stabilisation (Decho 2000; Underwood & Paterson 2003; de Brouwer *et al*. 2006; Lundkvist *et al*. 2007). The secreted material, in turn, acts as a carbon source for bacteria and invertebrates (van Duyl *et al*. 1999; Decho 2000; Middleburg *et al*. 2000).

Motility allows epipelic diatoms to move upward through the sediment for efficient light capture during the day and downward to resettle into more nutrient-rich environments at night (Saburova & Polikarpov 2003). Stimuli such as light (Cohn *et al*. 2004; Mitbavkar & Anil 2004; McLachlan *et al*. 2009; Ezequiel *et al*. 2015), temperature (Cohn *et al*. 2003), tides (Smith & Underwood 1998; Cohn *et al*. 2004; Mitbavkar & Anil 2004), nutrients (Cooksey & Cooksey 1988; Saburova & Polikarpov 2003; Witkowski *et al*. 2012), salinity (Abdullahi *et al*. 2006; Apoya-Horton *et al*. 2006), and toxic compounds (Coquillé *et al*. 2015) have been reported to provoke various movement responses.

In aquatic environments, chemosignaling and cell migration are important properties enabling the optimization of resource exploitation (Consalvey *et al*. 2004; Leynaert *et al*. 2009). Because of their circadian upward and downward migration within sediments, epipelic diatoms display a cyclic dependence on autotrophy on the photic zone and heterotrophy by osmotrophy in the aphotic lower levels (Saburova & Polikarpov 2003). The osmotrophic absorption of organic carbon in the lower aphotic sedimentary levels should be necessarily coupled to the ability to break down organic compounds at the cell surface or in the periplasmic space. Extracellular enzymes responsible for degrading proteins, peptides, and amino acids have been attributed both to heterotrophic bacteria (Hoppe *et al*. 2002) and phytoplankton, where cell surface amino oxidases (Mulholland *et al*. 2004; Palenik & Morel 1990a, b; Palenik *et al*. 1989; Pantoja & Lee 1994), peptidases (Mulholland *et al*. 1998, 2003; Mulholland & Lee 2009; Patel *et al*. 2000), and alkaline

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Table 1. Specific growth rates (r) and final cell densities of *Halamphora luciae* in f/2 (control) and A1–A5 culture media.

Culture media	$NaNO_3$ (μmol l^{-1})	Na_2HPO_4 (μmol l^{-1})	Growth rate (r)[1] (d^{-1})	Final cell density[1,2] (cells mm^{-2})
f/2	883	36	0.29 ± 0.01 A	417 ± 7.9 A
A1	883	9	0.37 ± 0.03 A	385 ± 7.9 B
A2	221	36	0.35 ± 0.02 A	373 ± 8.8 B
A3	0	0	0.32 ± 0.02 A	233 ± 5.2 C
A4	0	36	0.27 ± 0.02 B	224 ± 14.6 C
A5	883	0	0.23 ± 0.02 B	236 ± 20.5 C

[1] Data are expressed as media ± s, $n = 3$. Different capital letters indicate significant differences within each column according to one-way ANOVA and *post hoc* Tukey's test ($P < 0.05$).
[2] Cell density in stationary phase.

phosphatases (Dyhrman & Palenik 2003; Stoecker *et al.* 2005) have been described. In fact, the activity of extracellular hydrolytic enzymes has been used as an indicator of the nutritional status of the aquatic communities (Sala *et al.* 2001).

Halamphora luciae (Cholnoky) Levkov is a cosmopolitan, benthic, brackish-water species that was isolated from the mudflat of the Bahía Blanca estuary (Buenos Aires, Argentina).The object of the present work was to characterize the growth of the diatom under different N:P balances in axenic cultures and to relate motility and the induction of alkaline phosphatases (APs) and proteases to in vitro nutrient stress scenarios.

MATERIAL AND METHODS

Cells of *Halamphora luciae* were isolated from a mudflat located in Bahía Blanca Estuary (South Atlantic coast, 38°44′59.47″S, 62°22′51″W) by micropipette. To obtain axenic cultures from unialgal isolates, diatom cells were subjected to ultrasonication operating at a maximum frequency of 30 kHz in an ultrasonic bath for 60 s (Ultrasonic LC 30H, Elma, Germany) to separate algal clumps. Then, 1 ml of the cell suspension was incubated for 48 h with antibiotic solution containing penicillin G (12,000 units) and streptomycin (5 mg ml^{-1}), and finally thoroughly washed with autoclaved seawater (González *et al.* 1995). The strain has been maintained in our laboratory since 2013 in standard f/2 medium (Guillard 1975). Unialgal cultures are grown at 13°C in a 12:12 light:dark cycle using cool-white fluorescent light (16 μmol photons m^{-2} s^{-1}). Commercial seawater at a salinity of 30 practical salinity units (PSU) and pH of 8.0, enriched with nutrients according to Guillard (1975), was used in culture media preparation. Culture axenicity was periodically tested in bacteriological agar.

For cultures in different N:P ratios, exponentially growing cells in f/2 were repeatedly washed and resuspended in artificial seawater (30 PSU) for prestarvation for 7 days. To initiate the experiments approximately 34×10^3 cells were inoculated into wells containing 10 ml of culture media prepared with the N:P ratios indicated in Table 1. All cultures were carried out in triplicate.

In each well, algal cell density was estimated in at least 10 randomly selected optical fields (area 1 mm^2) in an inverted microscope (Olympus CK40, Tokyo, Japan) with the Arcano 9.0 video camera (Beijing, China). Cell counts proceeded on micrographs with the aid of TS View software. Exponential growth rate (r) was estimated by a least-squares fit to a straight line of logarithmically transformed data (Wood *et al.* 2005).

Subsamples of each treatment were processed following the standard methods for diatom analysis (Battarbee 1986). For scanning electron microscopy (SEM), clean valves were directly dried on copper tape and platinum coated for observation in a Gemini Zeiss DSM 982 microscope (Jena, Germany) equipped with a field emission gun and a secondary electron detector working at a distance of 4 mm and an accelerating voltage of 15 kV (CMA, FCEyN-UBA). For describing valve morphology we followed terminology suggested in Levkov (2009).

Environmental SEM (ESEM) was carried out with a XL30 Philips microscope (Hillsboro, Oregon, USA). The acceleration voltage was 20 kV and the chamber pressure 0.9 Torr. Cells were collected on round coverslips (10-mm diameter) that had been placed at the bottom of the culture vessels for 15 days. The coverslips were analyzed in triplicate.

For light microscopy, aliquots of clean valve suspension were dried on coverslips and mounted in Naphrax (Hasle & Fryxell 1970). Slides were examined under a Polivar Reichert-Jung light microscope (Vienna, Austria) equipped with differential interference contrast phase-contrast and digital photographic camera (Canon EOS 600D, Melville, New York, USA).

Cell biovolume and surface area were calculated after Sun & Liu (2003) in at least 100 valves. Because of their asymmetry, oxidized frustules almost always appear with the narrower ventral cingule and both raphes on the upper face. For morphometric calculations of the cymbelloid shape we measured the apical and the pervalvar axes. We considered the valve width as equivalent to the transapical axis.

Additionally, the length of 10–20 areolae was measured in the fourth to fifth striae counted from the proximal raphe fissure toward the poles in 40–50 valves. The number of areolae in 1 μm of the dorsal striae was recorded from 10 striae per valve.

The rate of cell motility was determined by measuring at least 10 randomly selected cells using image capture over a 20-s period. The speed was calculated by determining the distance between each start and stop position in the corresponding time interval and expressed as μm s^{-1}. All measurements are reported as mean ± standard error.

The kinetics of the production of soluble EPS (S-EPS) by *Halamphora luciae* was followed by sampling 200 μl every 4 days over a month to determine the extracellular total carbohydrate concentration according to Dubois *et al.* (1956).

To determine AP activity, cells from exponentially growing cultures were transferred to 100-ml beakers containing 40 ml of f/2 medium prepared without the addition of phosphate (A5). In different sets of assays P starvation was prolonged for 5, 15, and 25 days. Cells from A5 and control cultures in f/2 were harvested by centrifugation and resuspended in seawater buffered with 13 mM Tris-HCl pH 8.5.

Table 2. Morphometric observations in *Halamphora luciae* in f/2 (control) and A4 and A5 culture media.[1]

Culture media	Biovolume (μm^3)	Area (μm^2)	Area/volume	Areole length (μm)
f/2	359.8 ± 6.1	350.9 ± 2.1	0.98 ± 0.011	0.106 ± 0.005
A4	324.2 ± 5.4*	329.3 ± 1.7*	1.03 ± 0.01*	0.082 ± 0.006*
A5	367.8 ± 7.6	349.2 ± 2.8	0.97 ± 0.013	0.061 ± 0.004*

[1] Values represent media ± SE, $n = 100$.
* Significantly different ($P < 0.05$) with respect to f/2.

AP was measured using the artificial substrate 4-*p*-nitrophenylphosphate (*p*NPP) (Hernández & Whitton 1996). Enzymatic P_i cleavage was determined by reading absorbance at 410 nm, which indicates the formation of coloured *p*-nitrophenol (*p*NP) (molar extinction coefficient for *p*NP = 18.5 mM^{-1} cm^{-1}). The reaction mixture (1 ml) contained a known total protein content (as estimation of biomass, Fig. S1), 0.45 mM *p*NPP, and 13 mM Tris-HCl pH 8.5. The substrate was added at the onset of the reaction, which was stopped with 100 µl of 4 M NaOH every 10 min during the initial 90 min and every 30 min up to the 270-min end point. Determinations were carried out in triplicate. One AP unit is defined as the enzyme amount that catalyzes the formation of 1 mmol of *p*NP per minute at pH 8.5 and 37°C. Enzyme activity was expressed as AP units (µg $protein^{-1}$ μl^{-1}). Total protein was determined according to Lowry *et al.* (1951).

To determine proteolytic activity (PA), cells from exponentially growing cultures were transferred to beakers containing 30 ml of f/2 medium prepared without the addition of nitrate (A4). In a different set of assays N starvation was prolonged for 5, 10, and 20 days of incubation in the usual photoperiod and temperature. Cells were harvested by centrifugation and resuspended in 50 mM Tris-HCl pH 8.5 prepared in seawater. PA was measured using azocasein as a substrate, prepared according to Charney & Tomarelli (1947). The reaction mixture (400 µl) contained 150 µl of cell suspension and 250 µl of azocasein (15 mg ml^{-1}). Incubation proceeded at 37°C over 50 h of sampling at different time intervals. Enzymatic cleavage of the azo group was quantified by reading absorbance at 440 nm after precipitation with 500 µl of chilled trichloroacetic acid (5% w/v) and further alkalinization of the supernatant with 300 µl of 2 N NaOH (Pérez-Lloréns *et al.* 2003). Determinations were carried out in triplicate. Cells from cultures in complete f/2 medium were used as controls. One protease unit is defined as the enzyme amount that catalyzes the hydrolysis of 1 mmol of azocasein per minute at pH 8.5 and 37°C. Enzyme activity was expressed as PA units (µg $protein^{-1}$ μl^{-1}).

Statistical analyses were performed using InfoStat Release 2013. One-way analysis of variance (ANOVA) and repeated-measures ANOVA were used to determine statistical significance, and Tukey's honestly significant difference tests were used for *post hoc* treatment comparisons. The significance level was fixed at $P < 0.05$. For Mauchly's test we used the IBM-SPSS® Statistics package. Differences among cells grown in f/2 (control), A4, and A5 media were analyzed by multivariate ANOVA (MANOVA) for a set of response variables including biovolume, area, gliding speed, and areola length. Pillai's trace test was applied. Groups were differentiated by discriminant function analysis (DFA).

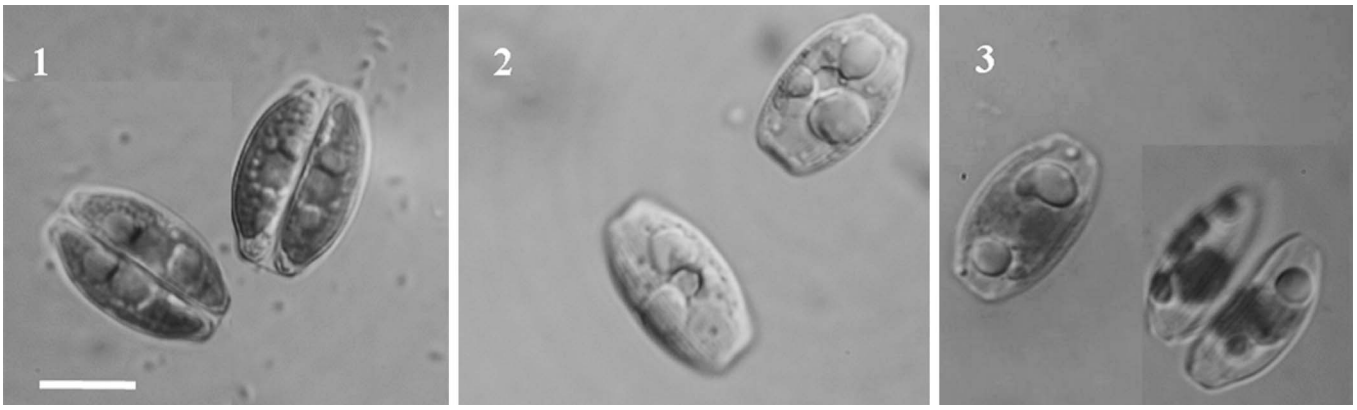
RESULTS

Growth and morphology of *Halamphora luciae* in nutrient-limited and -deprived media

The growth curves in well plates in all culture media were characterized by logarithmic and stationary phases (Fig. S2, supplementary data).The exponential growth rates (0.29–0.37 d^{-1}) did not differ significantly ($P > 0.05$) among f/2 control and assays A1, A2, and A3 (Table 1). But in A3, the exponential phase only extended from day 2 to 3, after an initial lag. P or N limitation and deprivation (A1–A5) reduced final cell yield significantly ($P < 0.05$) (Table 1).

In the assays where one of the essential nutrients was omitted (A4 and A5), exponential growth rates (0.23–0.27 d^{-1}) differed significantly ($P < 0.05$) from the control (Table 1). Only N-deprived cells showed significant ($P < 0.05$) reduction in cell biovolume and surface area when compared with the ones in f/2 and an increment in the area/volume ratio (Table 2). Cells grown under N deficiency appeared in clusters. When exposed to N and P deprivation, they were bleached, and had considerable reduction in chloroplast size together with lipid accumulation in oil bodies (Figs 1–3). Control cells also displayed oil bodies in the stationary phase, but chloroplasts were considerably bigger than in treatments.

In all culture conditions, cells of *Halamphora luciae* exhibited narrow and semilanceolate valves with capitate ends and straight ventral margins, slightly bent to the ventral side; their apical axes ranged between 15 and 40 µm and their pervalvar axes between 3.5 and 4.5 µm (Figs 4–10). The longest cells had valves with capitate ends, whereas the smallest had less protracted, rounded ends (Figs 4–10). The dorsal striae (20–22 in 10 µm) were parallel at the centre and became radiated toward the ends (Figs 11–15). Normal areolae were round to slightly transapically elongated, 0.1–0.13 µm long, except in culture media A4 and A5, where we found valves with punctiform areolae (0.03–0.05 µm in diameter). Internally, the row of areolae close to the raphe was separated from the others by a longitudinal rib. Under N (Figs 16–20) and P (Figs 21–25) deprivation, the number of areolae in the dorsal striae remained almost constant (two to three areolae in 1 µm) and reduction in areolae density was rare (Fig. 22). Areolae of cells growing under P and N deprivation were significantly ($P < 0.05$) smaller (0.03–0.05 µm in diameter) (Table 2 and Figs 26, 27). Under N deprivation, alterations in areola pattern of dorsal striae were observed, such as two punctiform areolae instead of a



Figs 1–3. Phase-contrast microscopy of *Halamphora luciae* cells from stationary phase cultures. Scale bar = 10 μm.
Fig. 1. f/2 (control) cultures.
Fig. 2. N-deprived f/2 (A4) cultures.
Fig. 3. P-deprived f/2 (A5) cultures.

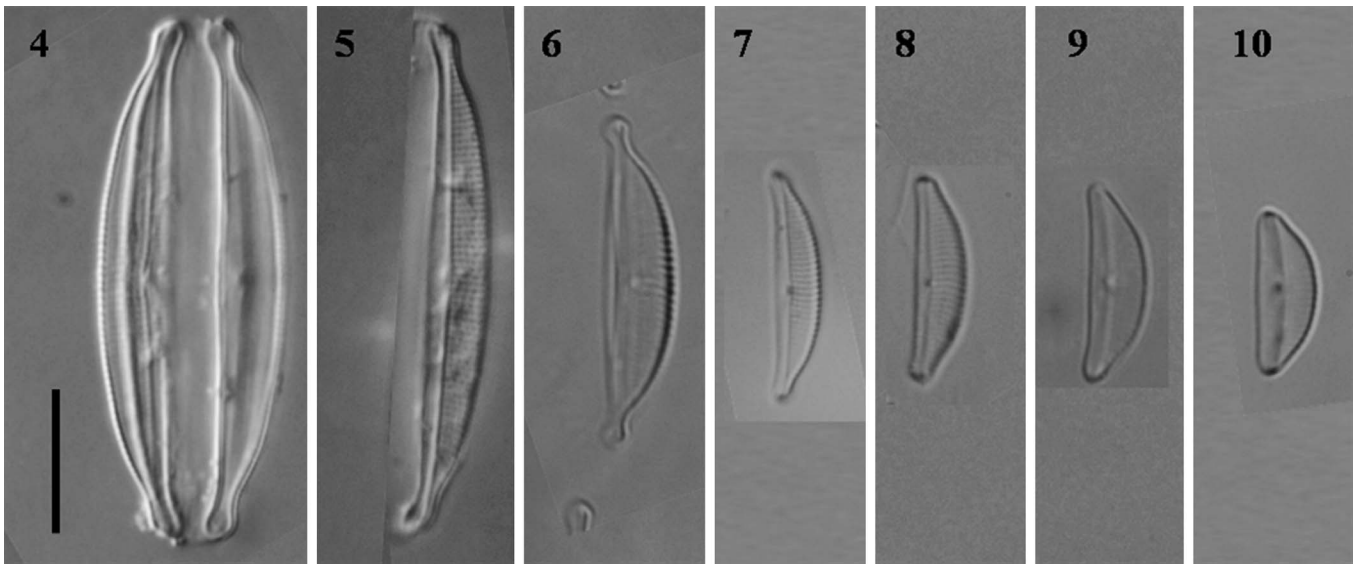
transapically elongated one (Figs 28, 29) and apparently fused areolae (Fig. 30).

Effect of nutrient limitation and deprivation on EPS secretion and the motility of *Halamphora luciae*

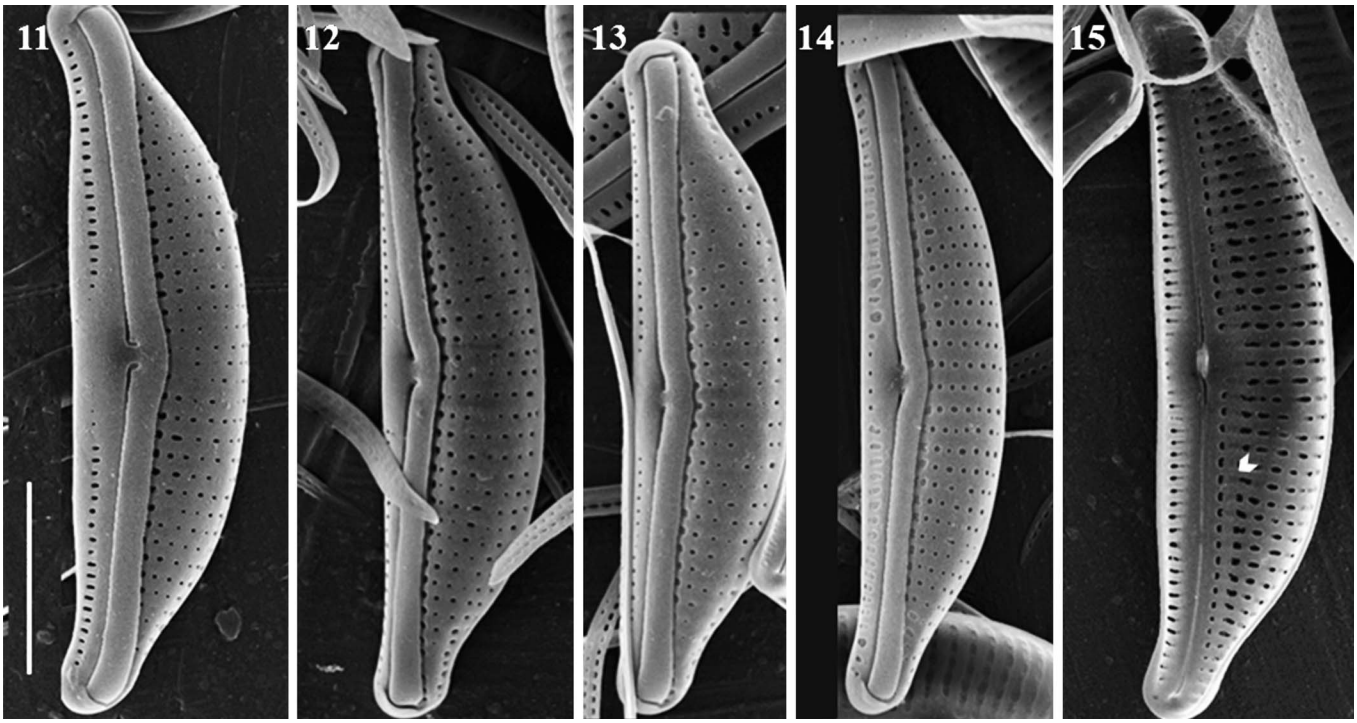
In general, there was a tendency to increase S-EPS secretion in stationary-phase cultures (Fig. 31). Both P limitation and deficiency enhanced S-EPS production significantly ($P < 0.01$) in N-replete media (A1 and A5) in the stationary phase (Fig. 31). On the other hand, a significant ($P < 0.05$) reduction of S-EPS was observed in both N limitation and deficiency, whether in presence or absence of P (A2–A4), especially in the late stationary phase (Fig. 31).

Cells of *Halamphora luciae* exhibited gliding movements. At times, one extreme of the cell remained attached to the bottom of the culture vessel while the other extreme projected upward, displaying a shunting movement in a plane (see Apoya-Horton *et al*. 2006). Finally, the free extreme became attached to the bottom and gliding was resumed. This pattern of movement was identical in all culture conditions.

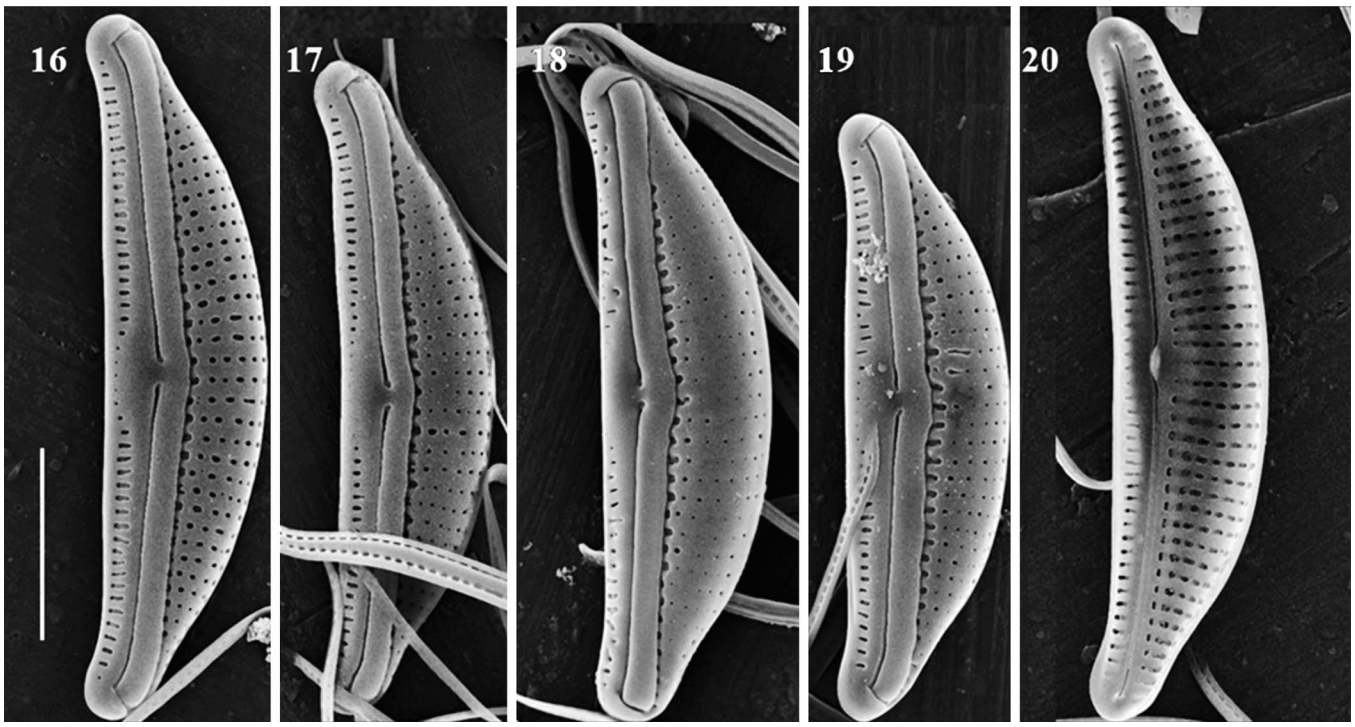
Except for cells grown in P-deficient medium (A5), algae in control and N-deficient (A4) medium showed a significant ($P < 0.05$) decline in gliding speed after 13 days (Fig. 32). Lower average speeds were registered for N-deficient cultures. As motionless cells appeared in all culture conditions, we compared the percentage of moving cells in randomly selected 0.2 mm^2 areas (Fig. 33). The lowest percentages of motile individuals were registered in N-starved diatoms, whereas significantly ($P < 0.05$) higher percentages of motility were observed in P-depleted medium. Moreover, the highest percentage of moving cells was found in P deficiency in the initial 13 days; in older cultures differences between the latter and the control were reduced.



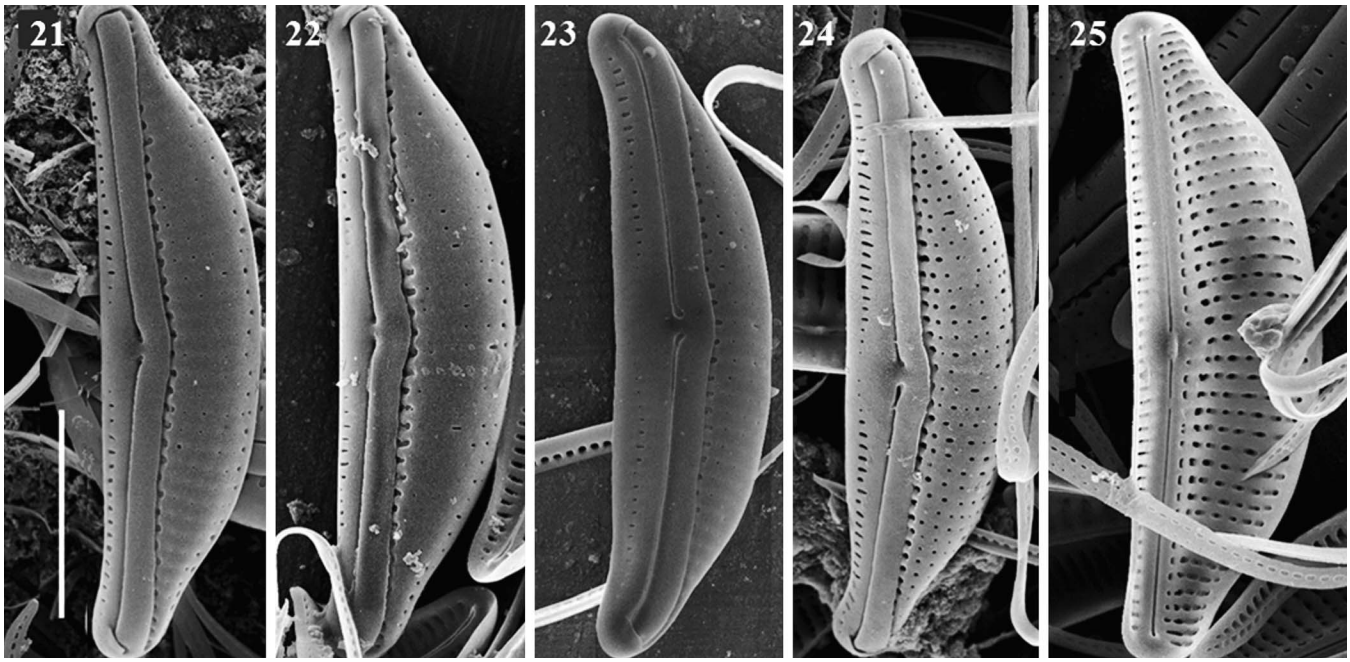
Figs 4–10. *Halamphora luciae*. Light microscope images. Scale bar = 10 μm.
Fig. 4. Frustule image.
Figs 5–10. Valve view of isolated valves showing morphological variability related to size reduction.



Figs 11–15. *Halamphora luciae* cultured in f/2 medium. SEM images. Scale bar = 5 μm.
Figs 11–14. External valve views.
Fig. 15. Internal valve view. Internal rib close to the raphe–sternum (arrowhead).



Figs 16–20. *Halamphora luciae* cultured in nitrate-depleted medium (A4). SEM images. Scale bar = 5 μm.
Figs 16–19. External valve views.
Fig. 20. Internal valve view.



Figs 21–25. *Halamphora luciae* cultured in phosphorus-depleted medium (A5). SEM images. Scale bar = 5 μm.
Figs 21–24. External valve views.
Fig. 25. Internal valve view.

ESEM images (Figs 34–36) indicated abundant mucilage secretion in gliding cells. Since living cells secrete mucilage through the raphes, the broad dorsal cingule is orientated to the upper face (Fig. 35). Mucilage accumulation in the raphe and in the extremes of the cells was denoted by refringent areas on top the frustules (Fig. 34). Mucilage trails were tether-like, with one extreme attached to the cells and the opposite ending in a holdfast-like entanglement of mucilaginous material (Figs 34–36). Remains of these tethers and more frequently holdfast areas were also observed detached from the cells.

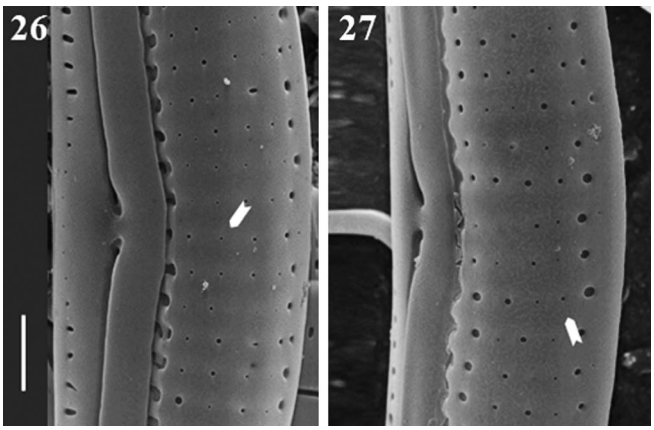
When applying a MANOVA test for the variables biovolume, area, gliding speed, and areola length, the differences were significant (Pillai's trace 0.09, $F_{8,50}$ 13.06, $P < 0.001$). The *post hoc* comparisons using the Hotelling (Bonferroni) indicated that the cells grown in f/2 and in N- and P-depleted media (A4 and A5) were in different groups ($P < 0.05$). DFA pointed out that the variability among media was explained mostly by the first canonical axis (78%) and mainly for gliding speed.

Incidence of phosphate starvation on AP activity

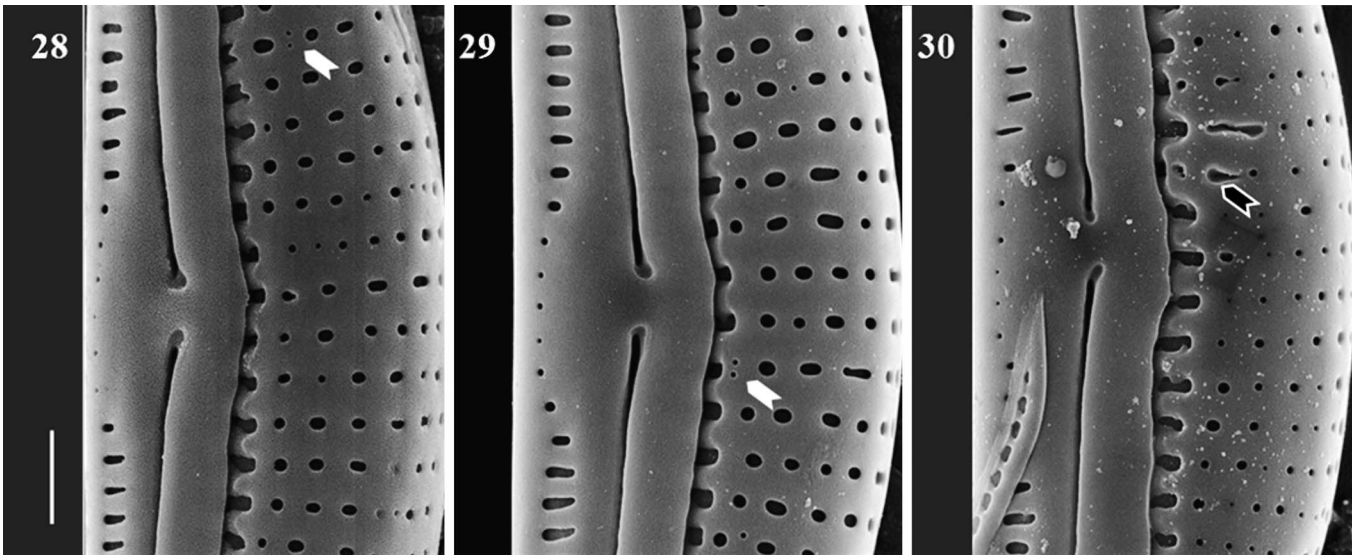
Cultures in beakers under P and N deprivation also showed reduced growth rate and final cell yield compared with f/2 controls, as observed in the well plates (Fig. S3). In general, P starvation induced AP activity. This induction was not so evident after a 5-day (exponential phase, Fig. S3) starvation (Fig. 37) but was very significant when there was an extended preincubation in absence of P for 10 (early stationary phase, Fig. S3) or 25 days (late stationary phase, Fig. S3) (Figs 38, 39). In both 10- and 25-day-old cultures in f/2 there were symptoms of P deficiency, since phosphatase activity was observed from the beginning of the incubation with the organic substrate.

Incidence of nitrate starvation on proteolytic activity

Proteolytic activity (Figs 40–42) differed depending on the length of the previous N starvation period. When starved for 10 days (early stationary phase, Fig. S3), N-deprived cultures showed significantly ($P < 0.05$ and $P < 0.01$) higher PA activity than controls after 20 h of incubation in the presence of azocasein (Fig. 41). When subjected to 5 days of N deprivation (exponential phase, Fig. S3), significant differences between control and treatment required longer incubation (40 h) with the organic substrate, with PA activity in N-deprived cultures higher only after 50 h ($P < 0.05$) (Fig. 40). However, if the previous starvation period lasted 20 days (late



Figs 26, 27. *Halamphora luciae* in phosphorus-depleted medium (A5). SEM images. Valves with punctiform areolae (arrowheads). Scale bar = 1 μm.
Fig. 26. Detail of Fig. 21.
Fig. 27. Another example of punctiform areolae.



Figs 28–30. *Halamphora luciae* cultured in nitrate-depleted medium (A4). SEM images. Scale bar = 1 μm.
Figs 28, 29. Valves presenting modified areolae: two punctiform areolae per striae instead of one (white arrowhead).
Fig. 28. Example of two punctiform areolae per striae.
Fig. 29. Detail of Fig. 16.
Fig. 30. Detail of Fig. 19. Valve with modified areolae: fused areolae (black arrowhead).

stationary phase, Fig. S3), significant differences between control and treatment also appeared after 40 h of incubation, but PA activity ($P < 0.01$) was always lower in N-deprived cultures (Fig. 42).

DISCUSSION

Exponential growth rates of *Halamphora luciae* in f/2 fell within the range informed for other benthic marine diatoms isolated from the same location (Popovich *et al*. 2012) and from the southern North Sea (Scholz & Liebezeit 2012). Changes in the exponential growth rate indicate the capacity for growth under limiting conditions. The incidence of nutrient limitation on growth rate depends on the species; in the case of *H. luciae* it seems to be negligible. Similar results were reported for *Coscinodiscus wailesii* Gran & Angst and *Cylindrotheca fusiformis* Reimann & J.C.Lewin (Magaletti *et al*. 2004; Armbretch *et al*. 2014). However, P limitation was reported to reduce growth rate in *Achnanthes brevipes* C.Agardh, *nom. illeg., C. fusiformis*, *Skeletonema costatum* (Greville) Cleve, and *Phaeodactylon tricornutum* Bohlin

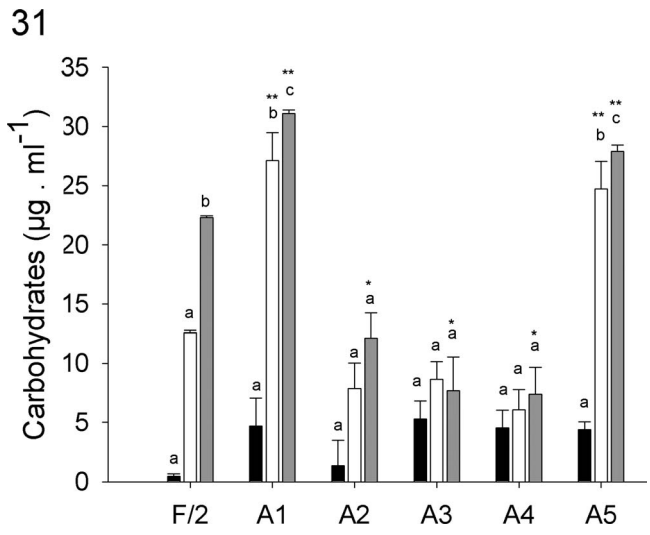


Fig. 31. Secretion of S-EPS (as total carbohydrates) in f/2 (control) and A1–A5 culture media. Dosages were performed at exponential (black bars), early stationary (light gray bars), and late stationary (dark gray bars) cultures. Different letters indicate significant *post hoc* differences ($P < 0.05$) among sampling times for each treatment. Asterisks represent significant differences (* $P < 0.05$; ** $P < 0.01$) with respect to f/2. Values represent mean ± s (n =3).

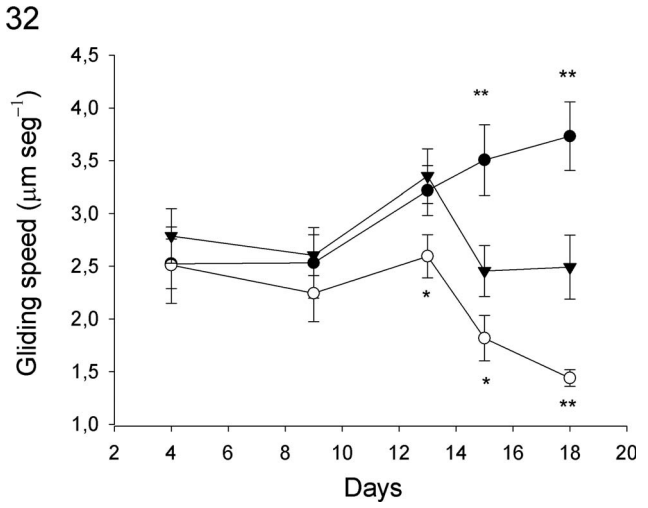


Fig. 32. Gliding speed of *Halamphora luciae* cultivated in f/2 (black triangles), A4 (white diamonds) and A5 (black circles) culture media. Asterisks indicate significant differences (* $P < 0.05$; ** $P < 0.01$) with respect to the control for each day. Values represent the mean ± s (n =10).

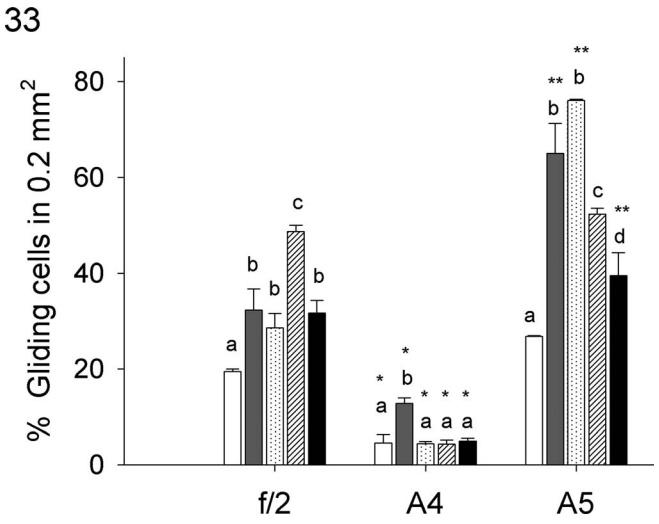
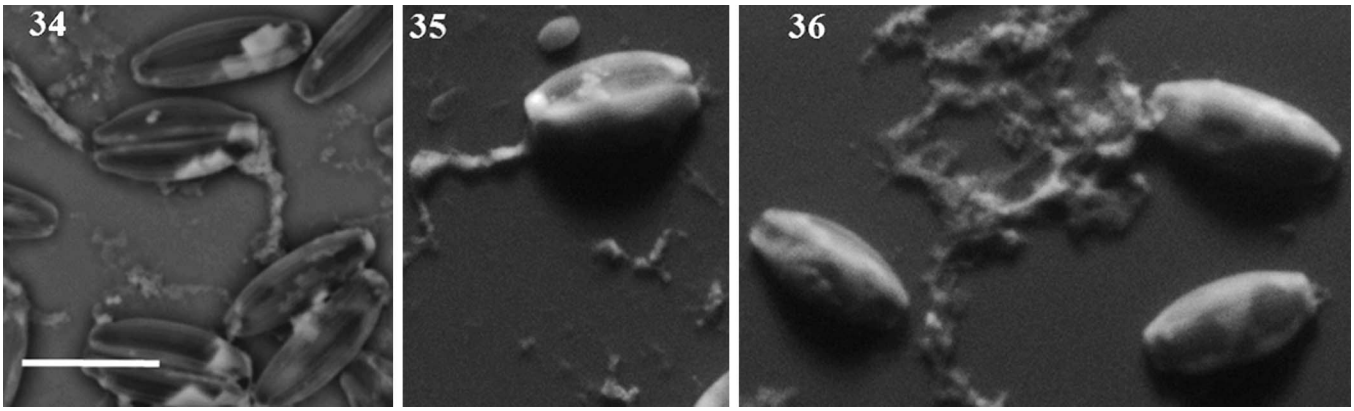


Fig. 33. Percentage of gliding cells in f/2, A4, and A5 culture media after 4 (white bars), 9 (gray bars), 13 (dotted bars), 15 (striped bars), and 20 (black bars) days' growth. Different letters indicate significant *post hoc* differences ($P < 0.05$) within each treatment. Asterisks represent significant differences (* $P < 0.05$; ** $P < 0.01$) with respect to f/2. Values are expressed as mean ± s (n =10).

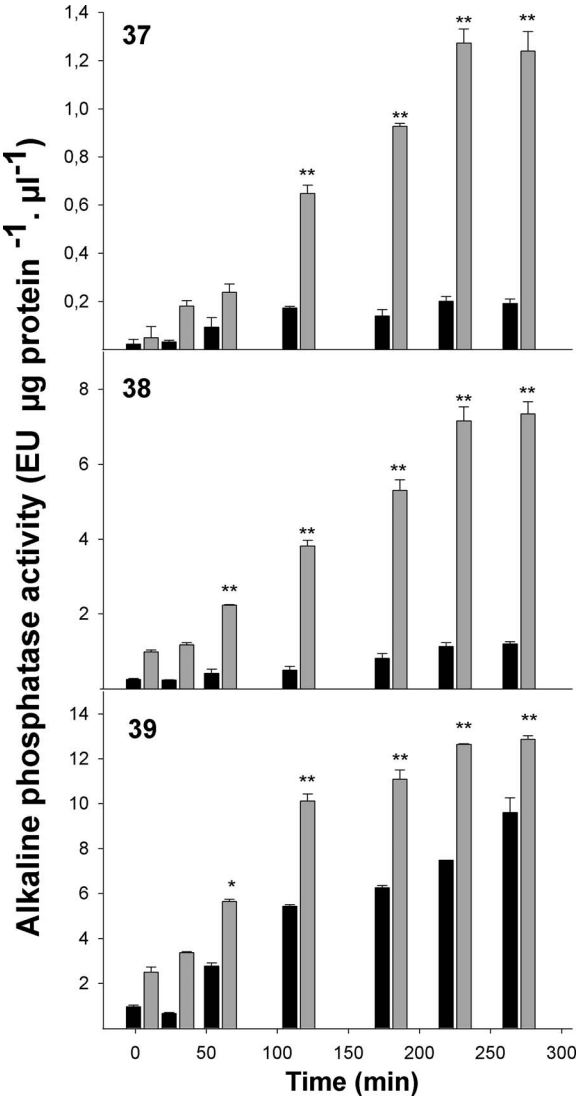
(Guerrini *et al*. 2000; Magaletti *et al*. 2004; Urbani *et al*. 2005; Abdullahi *et al*. 2006), whereas N limitation had a deleterious effect either on growth rate or final cell yield in *Chaetoceros affinis* Lauder (Myklestad & Haug 1972) and *Cylindrotheca closterium* (Ehrenberg) Reimann & J.C. Lewin (Alcoverro *et al*. 2000; Staats *et al*. 2000). Macronutrient limitation and deprivation in cultures of *H. luciae* led to a lower final cell yield, as reported for *C. closterium* and *Chaetoceros muelleri* Lemmermann (Staats *et al*. 2000; Leonardos & Geider 2004). Unchanged growth rate under nutrient limitation was attributed to luxury nutrient uptake (Eixler *et al*. 2006; Powell *et al*. 2009). Perry (1976) demonstrated that *Thalassiosira pseudonana* Hasle & Heimdal accumulated between 19 and 43% of cell P in polyphosphates.

Exposure to nutrient stress, either from culture aging or incubation in P- or N-deprived media, led to lipid accumulation and reduction in chloroplast size. *Halamphora luciae* shifted to the so called 'lipogenic phase', characterized by growth arrest, reduction of photosynthetic apparatus, and neutral lipid accumulation (Hu *et al*. 2008; Levitan *et al*. 2015), suggesting changes in plastid phospholipid and protein allocation. Recently, the breakdown of phospholipids, rather than mobilization of polyphosphate stores, were demonstrated to supply enough P to support several cell divisions in low-P conditions (Martin *et al*. 2011). In fact, replacement of phospholipids by sulfolipids or betaine lipids adjusting cellular P quota was observed in response to P limitation (Van Mooy *et al*. 2009; Dyhrman *et al*. 2012). Carbon allocation toward lipid accumulation was elucidated in the model diatom *Phaeodactylum tricornutum* (Alipanah *et al*. 2015; Levitan *et al*. 2015). Accordingly, *P. tricornutum* cannibalizes and remobilizes N in plastidial proteins toward energy storage, primarily in the form of triacylglycerols, by activating the assimilation of the nitrogen from the deamination of proteins simultaneously with the up-regulation of the urea cycle and energy-generating/anaplerotic reactions, such as the tricarboxylic acid cycle. As a consequence, diatoms may accumulate up to 60% of their dry weight in cytoplasmic oil drops of polyunsaturated fatty acids (Pulz & Gross 2004).

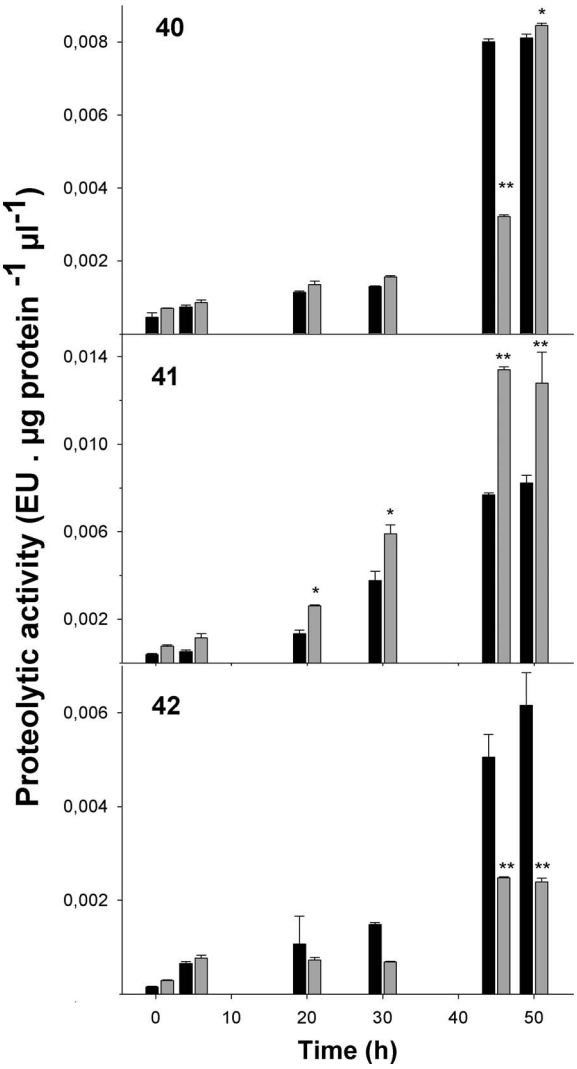
Inorganic nutrients must be absorbed from the surrounding medium over the cell surface, and once inside the cell, they are translocated to the site of use. Because the growth of a cell equals the difference between its uptake and expenditure, there is a cell size at which the net gain is optimized and, similarly, a maximum possible cell size where gain and expenditure are equal, preventing the cell from growing any further (Reynolds 1984). Estuarine mudflats, such as the Bahía Blanca Estuary, are usually nutrient-rich environments where diatoms frequently predominate among the microalgae of the benthic community (Saburova & Polikarpov 2003). In such locations, large cells that limit efficient nutrient exploitation would not be a drawback (Yamamoto *et al*. 2012). A frequent response in diatom populations to low nutrient concentrations is size reduction by increases in the surface:volume ratio (Sarthou *et al*. 2005). In the case of *Halamphora luciae*, only under N deprivation



Figs 34–36. ESEM observations of *Halamphora luciae* cells growing for 15 days in different culture media. Scale bar = 20 μm.
Fig. 34. Cells from f/2 medium.
Fig. 35. Cells from A4 medium.
Fig. 36. Cells from A5 medium.



Figs 37–39. Alkaline phosphatase activity in *Halamphora luciae* preincubated in f/2 (black bars) and A5 (gray bars). Enzymatic activity is expressed in µmol *p*NP μl^{-1} (µg protein)$^{-1}$. Asterisks represent significant differences (* $P < 0.05$; ** $P < 0.01$) with respect to f/2. Values are expressed as mean ± *s* (n =3).
Fig. 37. Five days of preincubation.
Fig. 38. Ten days of preincubation.
Fig. 39. Twenty-five days of preincubation.



Figs 40–42. Protease activity in *Halamphora luciae* pre-incubated in f/2 (black bars) and A4 (gray bars). Enzymatic activity is expressed in µmol azo group μl^{-1} (µg protein)$^{-1}$. Asterisks represent significant differences (* $P < 0.05$; ** $P < 0.01$) with respect to f/2. Values are expressed as mean ± *s* (n =3).
Fig. 40. Five days of preincubation.
Fig. 41. Ten days of preincubation.
Fig. 42. Twenty days of preincubation.

was there a reduction of biovolume and an increase of the area:volume ratio, as in *Stephanodiscus minutulus* (Kützing) Cleve & Möller (Lynn *et al.* 2000).

Though extracellular mucilage production is species specific (Penna *et al.* 1999; Chiovitti *et al.* 2003a, b; de Brouwer *et al.* 2006), nutrient limitation and the deviation of the N:P ratio above the classical Redfield ratio of 16 can stimulate extracellular secretion in marine diatoms (Myklestad 1995; Ruddy *et al.* 1998; Penna *et al* 1999; Alcoverro *et al.* 2000; Guerrini *et al.* 2000; Staats *et al.* 2000; Leandro *et al.* 2003; Magalletti *et al.* 2004; Underwood *et al.* 2004; Pistocchi *et al.* 2005; Abdullahi *et al.* 2006). The excretion would be favoured when the amount of fixed carbon exceeds the capacity of carbon storage within the cell (de Brouwer & Stal 2002). For *Halamphora luciae*, P limitation or absence seems to trigger extracellular carbohydrate production, whereas N absence or limitation in P-replete media reduces EPS secretion, as reported by Guerrini *et al.* (2000) for *Achnanthes brevipes*.

According to the Nielsen & Jahn (1999) definition, carbohydrate dosage in culture supernatants would correspond to soluble EPS fractions, whereas the observation of mucilaginous trails could be assigned to bound EPS ones. The light:dark cycle influences EPS secretion, making bound EPS highly light dependent, whereas soluble EPS is continuously released into the medium independent of light and dark conditions (de Brouwer *et al.* 2002). If a relationship is established between motility and the abundance of bound EPS, mucilage trails of bound EPS should be

more abundant in those cells exhibiting both higher speeds and percentages of gliding cells, such as control cells and P-starved ones. The use of ESEM allowed the visualization of trails without any drastic dehydration process, an important feature considering the hydrophilic nature of mucilages (Chiovitti *et al*. 2006). As the cells moved on, they left behind more diffuse, fraying trails indicating hydration and probable partial solubilization of polysaccharides and glycoproteins of the mucilage.

The horizontal gliding speeds of *Halamphora luciae* fell within the range previously found for other species (see Cohn & Disparti 1994; Consalvey *et al*. 2004). The movement modality was similar to *Cylindrotheca closterium* (Apoya-Horton *et al*. 2006), which might be linked to the fact that both strains were isolated from mudflat habitats. These authors attributed the shunting pivot movements of the cell to the perception of a chemical gradient to direct taxis under suboptimal conditions. Gliding movement would be resumed once the 'nutrient' route is established. In fact, nutrient deprivation seems to modify both velocities and percentage of moving cells of *H. luciae*. The tendency to cell aggregation in N-deprived cultures could be a consequence of lower motility rather than stickiness among cells through EPS production. Moreover, when under this condition, carbohydrate secretion was considerably lower. Witkowski *et al*. (2012) indicated that N and P deficiency diminished motility in *Navicula* sp., but in our case, P deficiency seemed to enhance both speed and percentage of moving cells, even in a late stationary phase.

Hydrolytic enzymes using organic substrates are produced to solve an anticipated shortage of N or P and rectify the nutrient imbalance. In *Halamphora luciae* the 'urge' to incorporate organic phosphate or nitrogen was stimulated by previous P and N starvation. Despite this, constitutive enzymes and other possible enzyme regulation mechanisms cannot be discarded. In fact, a low constitutive enzymatic activity was measured at the beginning of the reactions in all the assays. Evidence for both constitutive and substrate-induced enzymes has been reported for different algae (Hernández *et al*. 2002 and literature within). Taking into account that pNPP and azocasein are not membrane permeable (Lev *et al*. 2014), enzymatic activity is probably associated with either mucilage or the plasmalemma.

Induction response differed for both enzymes. Phosphatase activity was enhanced practically at the onset of the incubation in the presence of the organophosphate, whether P starvation had proceeded for 5, 15, or 25 days. Protease induction required at least 20 h (in controls and treatments) after the onset of the incubation with the azocasein probe, suggesting a longer time for enzyme activation, as for example, the requirement of *de novo* protein synthesis. Additionally, only a 10-day previous N starvation resulted in a significantly higher proteolytic activity in N-deprived cultures compared with the controls. But if N starvation was extended to 20 days, enzyme biosynthesis and induction in deprived cells was delayed with respect to controls. Carbohydrate fueling mitochondrial energy supply in these cells should be seriously affected by reduction of chloroplasts after prolonged N starvation, and in turn, this might also affect protein biosynthesis and cellular response to incubation in the presence of casein. Impaired photosynthesis and mitochondrial energy supply in N-deprived cultures also had a deleterious effect on cell motility mediated by the expulsion of glycoprotein mucilage trails.

We conclude that *Halamphora luciae* responds rapidly to P deprivation by increasing motility through EPS secretion and activation of AP. Only a limited N deprivation led to activation of protease, but the response to the absence of this macronutrient would mainly reside in reduction of both cell biovolume and motility.

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found online at http://dx.doi.org/10.2216/15-134.1.s1.

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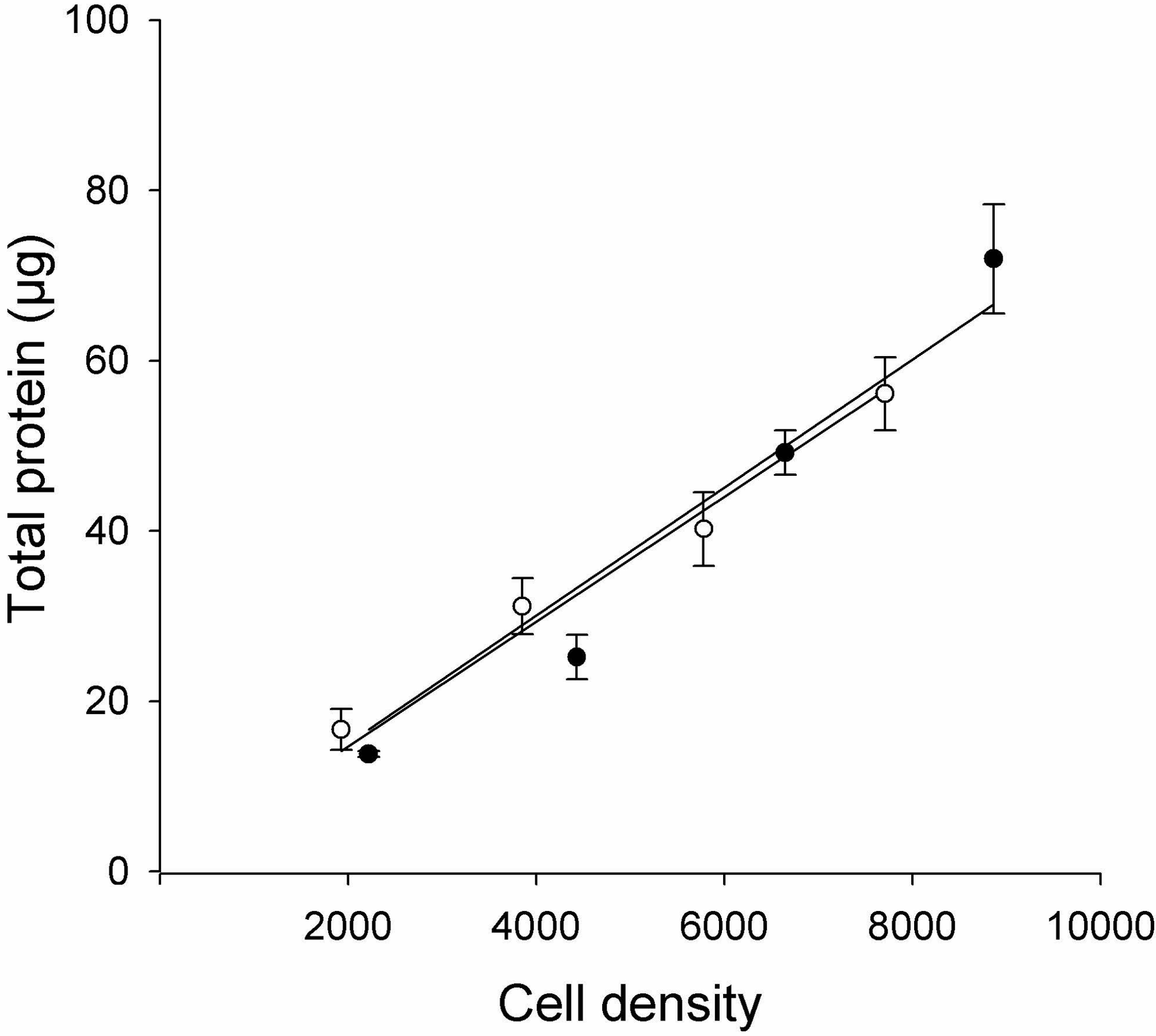
Fig. S1: Relation of total protein content (μg) and cell density of *Halamphora luciae* grown in f/2 (white diamond) and A3 (black square) during 12 days. The slope of the linear regression line was 0.0075 (R^2 0.95) for the f/2 medium and 0.0073 (R^2 0.97) for A3. Values represent the mean ± *s*, (*n* =3).

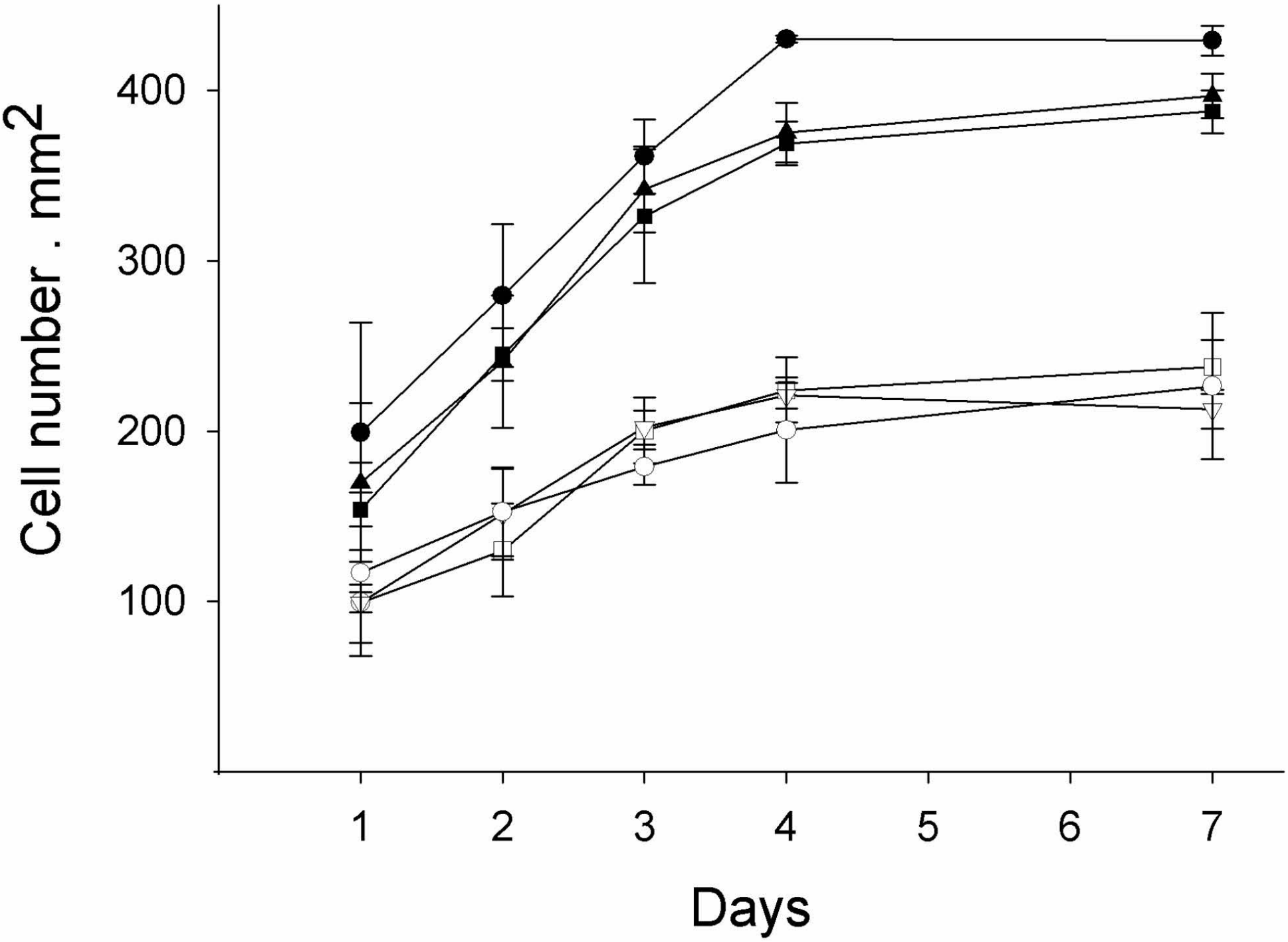
Fig. S2: Growth curves of *Halamphora luciae* in f/2 (black square) and f/2 with different N:P ratios. A1 (black triangle), A2 (black circle), A3 (white square), A4 (white triangle) and A5 (white circle). Values represent the mean ± *s* (*n* =3).

Fig. S3: Growth curves of *Halamphora luciae* in 100 ml beakers containing 40 ml of f/2 (black square), A4 (white triangle) and A5 (white circle) media. Values represent the mean ± *s* (*n* =3).

S1


100
80
60
40
20
0
Total protein (µg)
2000
4000
6000
8000
10000
Cell density

S2



S3