

SCIENTIFIC REPORTS



OPEN

Role of nutrient concentrations and water movement on diatom's productivity in culture

Ida Orefice¹, Margherita Musella¹, Arianna Smerilli¹, Clementina Sansone¹, Raghu Chandrasekaran^{1,2}, Federico Corato¹ & Christophe Brunet¹

Microalgal growth maximization is becoming a duty for enhancing the biotechnological fate of these photosynthetic microorganisms. This study, based on an extensive set of data, aims to revisit diatom's cultivation in laboratory with the objective to increase growth rate and biomass production. We investigated the growth ability and resource requirements of the coastal diatom *Skeletonema marinoi* Sarno & Zingone grown in laboratory in the conventional f/2 medium with aeration and in two modified conditions: (i) the same medium with water movement inside and (ii) an enriched medium with the same water movement. Results revealed that, by doubling the concentration of phosphate, silicate, microelements and vitamins, growth rate was successfully enhanced, preventing phosphate or silicate limitation in the f/2 culture medium. Yet, irrespective of the media (f/2 or enriched one), water movement induced an increase of growth efficiency compared to aeration, affecting nutrients' requirement and consumption by diatoms. This study is an important step for enhancing diatom biomass production, reducing its cost, as required in the blue biotechnology context.

The interest in maximizing microalgal production is nowadays growing since this group of photosynthetic microbes is greatly promising as natural sources of many products/processes useful for various biotechnological applications regarding the environment, energy, health, food, or cosmetics¹. Numerous researchers are facing this issue, applying different approaches, from genetic² to environmental manipulation, such as light modulation^{3,4}. Together with light and temperature, nutrients are one of the most important key drivers of phytoplankton growth^{5,6}. Microalgae require macro-, micronutrients and vitamins for growth. Macronutrients correspond to nitrogen (N) and phosphorus (P), while micronutrients correspond to trace metals (e.g. iron, manganese, cobalt, etc.). In addition, vitamins such as thiamine, biotin and cobalamin (vitamins B₁, B₇ and B₁₂, respectively) are also needed since some microalgal species are not able to synthesize them⁷. Diatoms require silicon (Si), which is involved in building the outer cell wall, or frustule⁸. Yet, silicate metabolism is tightly coupled with cell cycle⁹ and appears linked to respiration^{10,11}.

Nitrogen is required for biosynthesis of many molecules such as amino acids, nucleic acids, lipids and some sugars; the bulk of assimilated N being used for proteins and nucleic acids¹².

Phosphorus is a component of the backbone of DNA and RNA, with the elevated presence of sugar phosphate. RNA accounts for more than 50% of the total P-content of phytoplankton cells¹³, while RNA pool is extremely variable in cells¹⁴. Phosphorus is also present in phospholipids and ATP¹³ while cells can store P under the form of polyphosphate^{13,15}.

The molar stoichiometry of these elements in phytoplankton is known as "Redfield ratio"¹⁶, even though the relative contribution of these elements in cells varies with the demand for each of these components. Cell's requirement for these major elements depends on microalgal diversity^{15,17}, as well as on many environmental factors, such as light or temperature^{5,12,13,18–21}. Uptake and requirements of nutrients directly affect growth process, as reported in the Growth rate hypothesis (GRH)^{13,14,19,22}. The latter states "differences in organismal C/N/P ratios are caused by differential allocations to RNA necessary to meet the protein synthesis demands of rapid rates of biomass growth and development"¹⁴.

¹Stazione Zoologica Anton Dohrn, Istituto Nazionale di Biologia, Ecologia e Biotecnologie Marine, Villa comunale, 80121, Napoli, Italy. ²Present address: CAS in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai, 608502, Tamil Nadu, India. Correspondence and requests for materials should be addressed to C.B. (email: christophe.brunet@szn.it)

Medium	Water movement/aeration	Growth phase	Experimental strategy	Light conditions
f/2	Aeration (air-bubbling)	Exponential (n = 28) Stationary (n = 6) Death (n = 7)	Pre-acclimated	Blue (88, 150, 250, 450) Blue + red (150) Sinusoidal/Square Photoperiod: 12 h:12 h
f/2	Water movement (wave maker pump)	Exponential (n = 15) Stationary (n = 1) Death (n = 6)	Pre-acclimated	White (150) Sinusoidal Photoperiod: 12 h:12 h
Enriched medium	Water movement (wave maker pump)	Exponential (n = 101) Stationary (n = 12) Death (n = 42)	Pre-acclimated (n = 122) Shift (n = 33)	White (10, 88, 150, 300, 600) blue (150); red (150); green (150) Photoperiod: 12 h:12 h; 24 h:0 h; 0 h:24 h Sinusoidal/Square

Table 1. Experimental data set. Light intensity in brackets is in $\mu\text{mol m}^{-2} \text{s}^{-1}$. Sinusoidal is for sinusoidal light distribution, square is for wave square light distribution. Blue, red, green are for monochromatic blue, red, green wavelengths, respectively; blue + red is for blue light condition over imposed with red light peaks. White is for white light composed by blue, red and green (50, 5, 45%, respectively). n is the number of data for each class.

Another crucial aspect for microalgal cultivation is the shear stress²³ and small-scale turbulence^{24–26}. Mixing circulate fluid is essential for microalgal cultivation helping to maintain homogeneous suspension and ensures that microalgal cells have access to the resources, such as light or nutrients. Aeration is usually applied to microalgal culture supplying CO_2 , removing O_2 produced by photosynthesis and helping in fluid circulation. While air bubbling can regulate pH variations during cultivation^{27,28}, it is also known to be detrimental to microalgal cells²⁹.

Starting with the observations of growth limitation by nutrient (P and/or Si) of the coastal diatom *Skeletonema marinoi* Sarno & Zingone, our study aimed to enhance growth rate of this nutritionally-rich species^{3,30}, relevant for aquaculture purposes^{31–34}. The objectives of this study are (i) to investigate the modulation of growth rate and (ii) to address the GRH quantifying the macronutrient uptake of this species grown in the conventional f/2 medium and in an enriched medium. The third objective is to enhance growth of this species addressing the question of mixing, comparing aeration with water movement created by an aquarium wave-maker pump.

Growth rate and uptake of nitrate, phosphate, and silicate has been estimated during 21-laboratory microcosm experiments carried out on this diatom. Cellular RNA content enriched the dataset and allowed to investigate the link between growth rate, nutrient uptake and physiological state of the cells.

Materials and Methods

The model species *Skeletonema marinoi*. The species *S. marinoi* is very abundant during the spring bloom in temperate waters when its concentration can reach millions of cells per litre in the photic zone. *Skeletonema marinoi* (CCMP 2092), a cosmopolitan centric diatom, was used as a model species since its high growth capacity, its biotechnology interesting biochemical profile^{3,30} and relevant role for aquaculture applications^{31–34} and the information already available on the biology of this species^{3,4,35},

Experimental data set. Our study is based on 291 data, obtained from 21 experiments conducted on the coastal centric diatom *S. marinoi* (Table 1). Experiments were conducted on axenic cultures. All experiments were carried out at 20 °C with autoclaved seawater, pre-filtered through a 0.7 μm GF/F glass-fiber filter (Whatman™, Whatman International Ltd, Maidstone, UK). Light was provided by a custom-built LED illumination system (European patent registration number: EP13196793.7), allowing to modulate the spectral composition and light intensity³⁶. Light intensity was measured inside each flask by using a laboratory PAR 4 π sensor (QSL 2101, Biospherical Instruments Inc., San Diego, CA, USA).

The first set of data corresponded to *S. marinoi* cultivated in the classical f/2 medium for diatom's cultivation³⁷ with an addition of silicate. The second experimental data set originated from *S. marinoi* cultivated in an enriched medium, characterized by twice the concentrations of phosphate, silicate, metals, and vitamins compared to the conventional f/2 medium.

Two conditions of physical motion in the fluid were compared: aeration (air bubbling) vs. water movement using an aquarium wave maker pump (Sunsun, JVP-110) (Table 1).

The dataset was discriminated respect to the growth phases: exponential phase, stationary phase or death (Table 1), and the different light conditions in term of light intensity, spectral composition and distribution over time were taken into account. Moreover, we separated the experiments carried out on pre-acclimated cells to the experimental light condition vs. shifted cells from a pre-acclimation light to the experimental light conditions (Table 1).

Nutrient analysis and nutrient uptake rate estimation. Samples for determining nutrient concentrations were collected in 20 mL polyethylene vials, and quickly frozen and stored at –20 °C. Ammonium, nitrate, nitrite, silicon and phosphate concentrations were determined using a Technicon Auto Analyzer following classical methods³⁸.

Nutrient concentrations sampled every day were therefore used to estimate the daily uptake of nutrients by cells:

$$\text{Nu} = \frac{N_n - N_{n-1}}{C_n - C_{n-1}} \quad (1)$$

where Nu is the nutrient uptake ($\text{pmol cell}^{-1} \text{ day}^{-1}$), N_n the nutrient concentration at day n and C_n the cell concentration at day n.

Cell concentration and growth rate. To assess cell density, 2 mL of cell suspension were collected from each flask and fixed with Lugol's iodine solution (1.5% v/v). One mL of this solution was used to fill a Sedgewick Rafter counting cell chamber. Cells were then counted using a Zeiss Axioskop 2 Plus light microscope (Carl Zeiss, Göttingen, Germany).

The growth rate was estimated from cell concentration measurements using the following equation:

$$\mu(\text{d}^{-1}) = \frac{\ln(C_{n-1}/C_n)}{t_n - t_{n-1}} \quad (2)$$

where μ is the growth rate, C_n and C_{n-1} are cell concentrations (mL^{-1}) at day n (t_n) and day n (t_{n-1}).

Assuming that every individual cell doubles with every cell cycle, we estimated the proliferation of cells by the equation:

$$C_n = C_{n-1} 2^{ft} \quad (3)$$

where C_n is the cell concentration at day n, C_{n-1} is the cell concentration at day n - 1, and f is the frequency of cell cycles per unit of time.

Intracellular phosphorus partition estimation. The P uptake rate per cell (see above, $\text{pmol cell}^{-1} \text{ day}^{-1}$) was transformed in content of P per cells (pg cell^{-1}), using the phosphorus molar weight, assuming that all acquired phosphorus is internally used by cells. With the aim to discriminate the phosphorus allocation in cells, we proceeded in the following way: we evaluated the P-containing RNA content applying the data $P = 0.091 \text{ g dry g RNA}^{-1}$ ¹² to the cellular RNA concentration (see below). Then, we obtained the no-RNA-P content per cells (removing the P-containing RNA from the total P content per cell estimated previously). Two other pools of cellular P can be discriminated¹²: the total inorganic phosphorus and the other P-containing compounds (i.e., excluding RNA and inorganic P pools). From the data reported in Geider and La Roche¹², we applied a value of 20% on the total no-RNA-P content to obtain the total inorganic phosphorus in cells while the other 80% accounts for the other P-containing compounds¹².

RNA analysis. Fifty mL of each of the triplicates were centrifuged at 4000 rpm (3399 g) for 20 min at 4 °C (DR15P centrifuge, B. Braun Biotech International, Melsungen, Germany). The supernatant was discarded and the pellet was transferred to a 2 mL Eppendorf tube and centrifuged at 14000 rpm (21952 g) for 15 min at 4 °C (5417 R centrifuge, Eppendorf, Hamburg, Germany). The pellet was re-suspended in 800 μL of TRIzol (Invitrogen, Carlsbad, CA, USA), incubated for 2–3 min at 60 °C until it was completely dissolved. Samples were frozen in liquid nitrogen and kept at -80 °C until analysis. The total RNA has been extracted following the already described procedure³⁹. DNase treatment was carried out using DNase I recombinant, RNase-free (Roche, Basel, Switzerland). Then, total RNA sample was purified and concentrated using RNeasy MinElute Cleanup Kit (Qiagen, Venlo, Netherlands) and eluted in 20 μL RNase-free water. Concentration of resulting RNA was evaluated by absorbance at 260 nm (ND-1000 Spectrophotometer; NanoDrop Technologies, Wilmington, DE, USA) and RNA integrity was checked by agarose gel electrophoresis.

Statistical analysis. Statistical analysis was performed using Past 3⁴⁰. We performed calculations of mean, standard deviation, variance, the coefficient of variation (CV). Mann-Whitney U test was applied to compare the data between different discriminated groups. Spearman correlation has been applied to analyse the trend between our variables separating or including different experiments.

Results and Discussion

Although it is known that light significantly affects growth capacity and thus nutrient requirement^{6,11,12}, our study did not depicted large effects of light on nutrient uptake. Nitrate (NO_3^-) and silicate (SiO_4^{4-}) uptake did not significantly vary under the different light conditions, while under prolonged darkness NO_3^- uptake decreased significantly ($p < 0.001$). On the opposite, phosphate (PO_4^{3-}) uptake increased under low light compared to moderate and high light ($p < 0.01$). Since the very circumscribed effects of light and, in order to prevent a bias in the further analysis, we excluded the data conditioned by light effect from the dataset.

Growth and nutrient uptake of *S. marinoi* grown in f/2 medium with aeration. Our study reveals that in the conventional f/2 medium used for diatom's cultivation⁴¹ cells reach the stationary phase in parallel with the depletion of P and/or Si in agreement with previous studies^{36,42}. PO_4^{3-} concentration dropped down to $< 0.1 \mu\text{mol L}^{-1}$ (Fig. 1A,B) when *Skeletonema marinoi* reached a concentration of 500,000 cells mL^{-1} . Also, SiO_4^{4-} concentration strongly lowered, reaching $< 0.5 \mu\text{mol L}^{-1}$ (Fig. 1C), while NO_3^- decreased along the growth curve, without becoming limiting (Fig. 1D). NO_3^- concentration decreased in parallel with an increase of nitrite (NO_2^-) and ammonium (NH_4^+) concentrations (Fig. 2A,B), while the increases of NO_2^- and NH_4^+ concentration were significantly correlated together ($p < 0.01$; Fig. 2C). The low decrease in NO_3^- and the strong increase of NH_4^+ along the growth curve indicates that these elements are probably not the main N source of *S. marinoi*. It is surprising since NH_4^+ is considered as the preferred N source by many microalgae thanks its reduced state and energetically favourable assimilation⁴³.

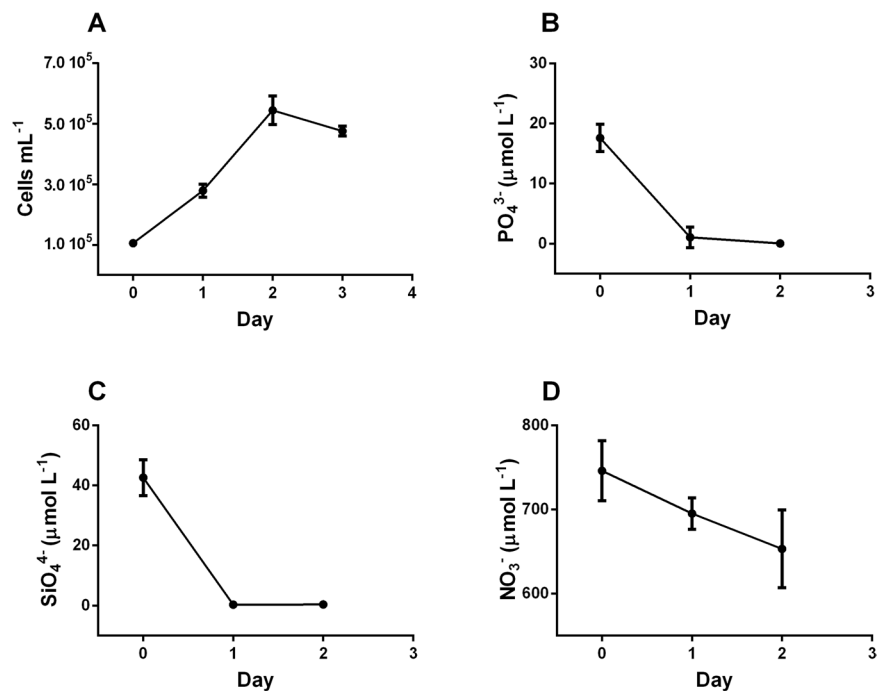


Figure 1. Cell concentration (A), cells mL⁻¹ and PO₄³⁻ (B), SiO₄⁴⁻ (C), and NO₃⁻ (D) concentrations (μmol L⁻¹) along the exponential growth phase in the original *f/2* medium with aeration.

In culture medium, organic nitrogen coming from microalgal excretion occurring during death or lysis or also during productive growth phase can be a source for NH₄⁺ production⁴⁴. Urea is known as one of the main organic sources of NH₄⁺⁴⁵. Since the high biomass concentration and the elevated growth and division rate, we expect a high concentration of organic nitrogen (e.g. urea, amino acids) in the medium that in turn can be re-used by microalgae^{46,47}. This hypothetical cartoon matches with the low decrease of NO₃⁻ with time during high growth rate phase and agrees with results demonstrating that NH₄⁺ in the medium has a negative effect on nitrate reductase enzyme (that catalyses the NO₃⁻ reduction to NO₂⁻) at both transcriptional and posttranscriptional levels in *Chlamydomonas reinhardtii*^{20,48,49}.

These assumptions require further studies to better understand the biochemical cycle of N in diatom's cultivation, since its relevance for the optimization of microalgal cultivation conditions.

These studies would focus on understanding the composition of the organic dissolved matter released in the medium, its evolution in time along the growth curve and on which nitrogen form diatoms do prefer and use when cultivating.

Nutrient uptake varied with growth rate (Table 2). Growth rate increase induced a decrease of NO₃⁻ uptake ($p < 0.05$) and a PO₄³⁻ uptake increase ($p < 0.05$). SiO₄⁴⁻ uptake was almost stable. During the population death phase (negative growth rate class, Table 2), PO₄³⁻ and SiO₄⁴⁻ uptake significantly lowered ($p < 0.01$) conversely to NO₃⁻ uptake, which remained stable compared to low positive growth rate class. The N:P ratio strongly decreased with the increase of growth rate ($p < 0.01$), while the N:Si ratio decreased slightly from low to high growth rate and the P:Si ratio little increased (Table 2). P concentration allocated to RNA was stable in the two positive growth rate classes (Table 2), while the total no-RNA-P content tended to increase, mainly due to the P-containing non-stored products (e.g., DNA, ATP, phospholipids).

On the role of water movement. Using a wave marker pump instead of air-bubbling, growth rate was significantly higher ($p < 0.01$) compared to aeration (Fig. 3A), as reported by other studies^{24,25,42,50}. While aeration ensures the exchanges between the medium and air thanks the addition of gas in the culture, water movement prevents sedimentation of the algae, ensures that cells are equally exposed to light²⁶ and that they continuously explore new microscale environments^{24,25} with renewed nutrients composition/concentration, as well as improving gas exchange between the medium and the air. Dell'Aquila and co-authors⁴² hypothesized a better physiological state of cells grown under mixing compared to stagnant condition. Indeed, turbulence increases the chain length – used as proxy of a healthy physiological state⁴² – of the diatom *Chaetoceros decipiens*, and induces energy storage pathways like fatty acid biosynthesis⁵¹.

In parallel to growth rate enhancement, requirement in NO₃⁻ and PO₄³⁻ also increased. As in the previous culture condition, PO₄³⁻ became limiting with concentration dropping down to < 1 μmol L⁻¹ (Fig. 3B), while SiO₄⁴⁻ concentration decreased (Fig. 3C) but did not reach low values as observed during cultivation with aeration (Fig. 1C). NO₃⁻ concentration remained high and was not limiting for growth (Fig. 3D).

Regrouping the data set in three growth rate classes (Table 3), it is noteworthy that SiO₄⁴⁻ uptake significantly decreased ($p < 0.01$) with increasing growth rate, while PO₄³⁻ uptake slightly increased. NO₃⁻ uptake remained

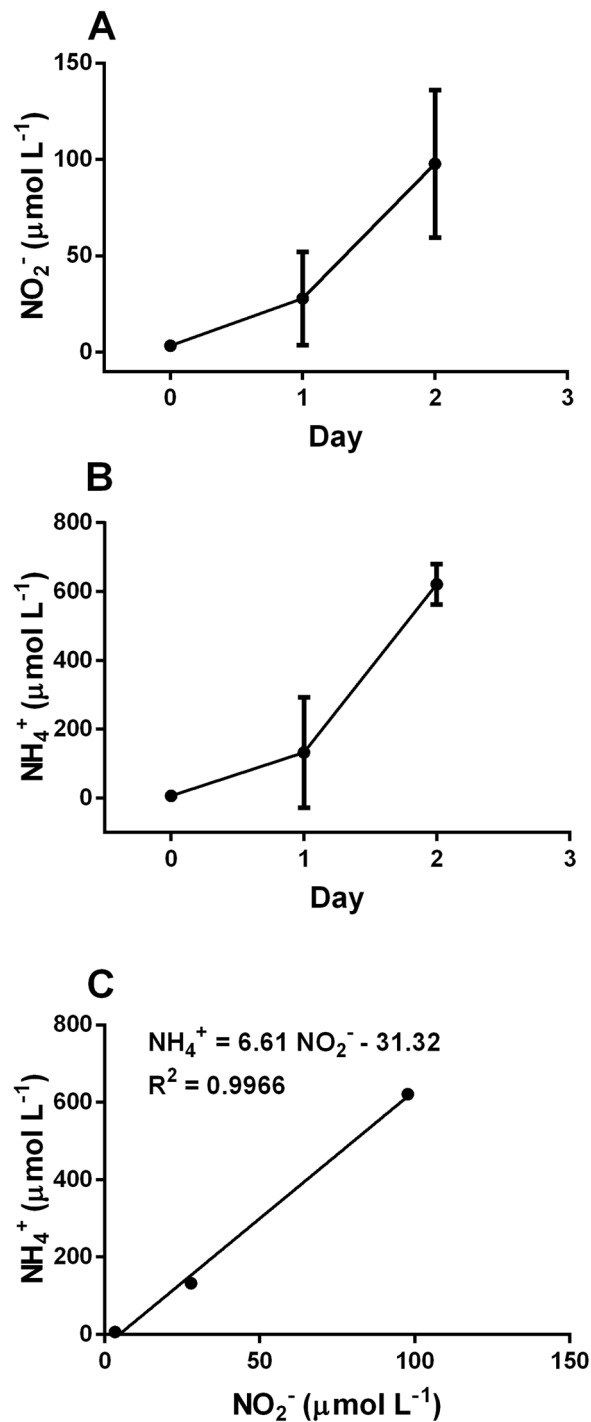


Figure 2. NO_2^- (A) and NH_4^+ (B) concentrations ($\mu\text{mol L}^{-1}$) along the exponential growth phase in the original *f/2* medium with aeration; (C) correlation between NO_2^- and NH_4^+ concentrations during the exponential growth phase.

almost stable. The N:P ratio was significantly higher in the two positive growth rate classes compared to dying population class ($p < 0.05$), while it decreased with increasing growth rate ($p < 0.05$). N:Si ratio increased in parallel to growth rate ($p < 0.05$, Table 3), on the contrary to the previous observations reported in culture with aeration (Table 2). The P:Si ratio strongly increased with growth rate ($p < 0.05$, Table 3) as already found in the previous condition (*f/2* with aeration).

Comparing aeration and water movement, the trend of PO_4^{3-} uptake with increasing growth rate is similar. Conversely, in efficiently growing cells, water movement induces a lowering of SiO_4^{4-} uptake, as reported in another diatom⁵² and an increase of NO_3^- uptake conversely of what it has been observed in the high growth rate class of cells submitted to aeration. Although the role of turbulence on microalgal nutrient acquisition has been

Growth rate	N	P	Si	N:P	N:Si	P:Si	P-RNA	P-storage	P-no storage
-0.70 d ⁻¹ to 0.00 d ⁻¹ (n=7)	0.37 ± 0.03	0.008 ± 0.001	0.01 ± 0.002	48.16 ± 4.80	25.46 ± 2.82	0.53 ± 0.07	0.017 ± 0.007	0.044 ± 0.02	0.176 ± 0.08
0.04 d ⁻¹ to 0.30 d ⁻¹ (n=12)	0.39 ± 0.12	0.01 ± 0.006	0.1 ± 0.01	27.67 ± 4.98	3.91 ± 0.92	0.14 ± 0.05	0.044 ± 0.01	0.12 ± 0.02	0.48 ± 0.09
0.40 d ⁻¹ to 1.40 d ⁻¹ (n=22)	0.19 ± 0.06	0.04 ± 0.02	0.07 ± 0.03	5.10 ± 1.86	2.82 ± 1.02	0.55 ± 0.23	0.038 ± 0.03	0.174 ± 0.14	0.696 ± 0.46

Table 2. Nutrient uptake (pmol cells⁻¹ d⁻¹) and ratios between N:P, N:Si and P:Si uptaken by cells grown in f/2 medium with aeration. P-RNA (pg) is for P content allocation in RNA (using P = 0.091 g dry g RNA⁻¹)¹². P-storage (pg) is for P content allocation in reserve compounds (inorganic phosphate) and P-no storage (pg) is for P content allocation in other phosphorus-containing compounds. n is the number of data for each class of growth rate.

Growth rate	N	P	Si	N:P	N:Si	P:Si
-2.45 d ⁻¹ to 0.00 d ⁻¹ (n=6)	0.37 ± 0.02	0.09 ± 0.01	0.30 ± 0.06	4.18 ± 0.47	1.23 ± 0.18	0.29 ± 0.05
0.07 d ⁻¹ to 0.50 d ⁻¹ (n=3)	0.75 ± 0.19	0.04 ± 0.003	0.48 ± 0.05	17.6 ± 2.96	1.54 ± 0.28	0.09 ± 0.01
0.60 d ⁻¹ to 1.20 d ⁻¹ (n=13)	0.61 ± 0.04	0.06 ± 0.03	0.16 ± 0.08	8.95 ± 2.88	3.64 ± 1.04	0.40 ± 0.21

Table 3. Nutrient uptake (pmol cells⁻¹ d⁻¹) and ratios between N:P, N:Si and P:Si uptaken by cells grown in f/2 medium with water movement. n is the number of data for each class of growth rate.

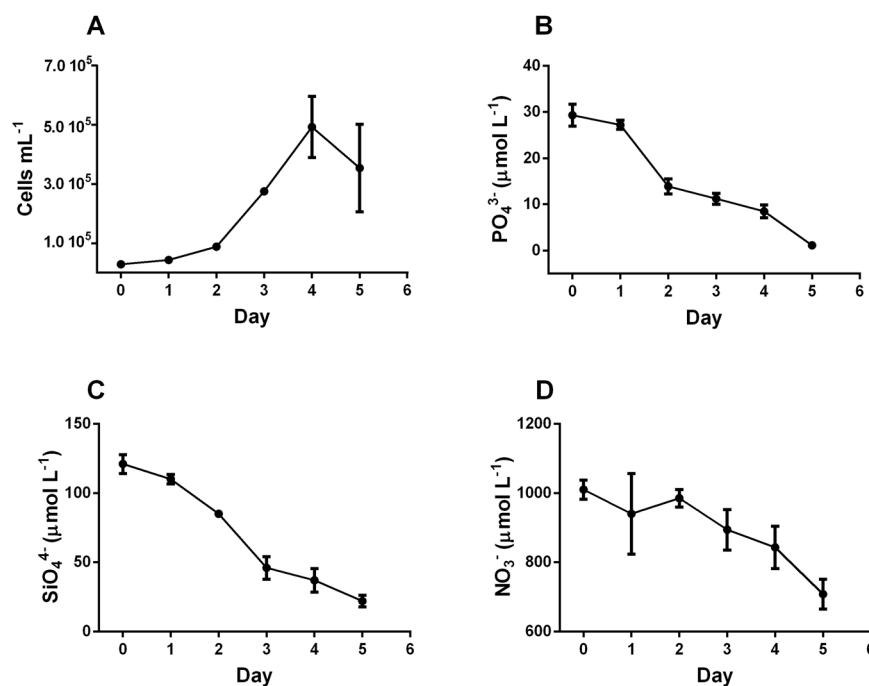


Figure 3. Cell concentration (A), cells mL⁻¹ and PO₄³⁻ (B), SiO₄⁴⁻ (C), and NO₃⁻ (D) concentrations (μmol L⁻¹) along the exponential growth phase in the original f/2 medium with water movement.

the object of different studies^{23–25}, few data on the three main nutrients together are available. More commonly, nutrient acquisition increases^{46,50,52} even though depending on algal species²⁵, turbulence strength^{24,25} and the type of nutrients⁵². Our study also shows that differences in nutrient acquisition between different kinds of fluid circulation are strongly influenced by growth rate. Indeed, the significant link of NO₃⁻ or PO₄³⁻ acquisition to growth rate has been reported in some studies³⁰ (also demonstrating the little influence of light condition compared to growth rate) by contrast to SiO₄⁴⁻ acquisition^{30,53}. Therefore, SiO₄⁴⁻ uptake and its cellular use respond to other external or internal signals that the two other elements. As discussed in Spitzer⁵³, Si requirement and metabolism in diatoms is variable and depends on species and cell size. Moreover, Levitan *et al.*⁵⁴ report that Si-starvation is not physiologically growth damaging as N starvation. Another study⁵³ reports that the silica present in frustules of two diatoms species is modulated with respect to the Si concentration in the media, without any variations of growth rate between high or low Si content. This suggests that cells efficiently growing preferably use internal Si pool for computing cell cycle progression and division, while Internal Si pool and its participation in the cellular metabolism respiration¹¹ is controversial⁹.

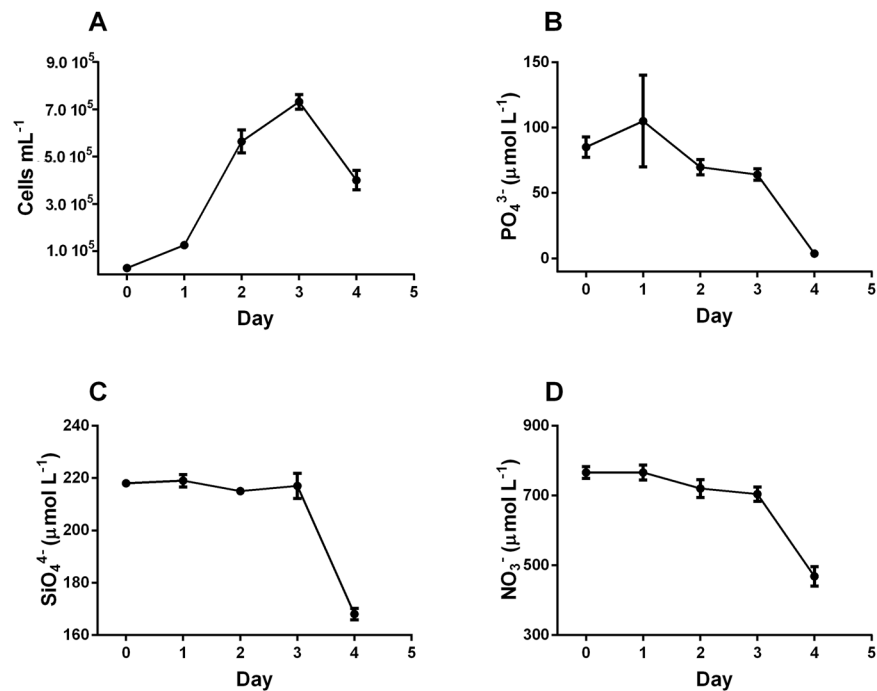


Figure 4. Cell concentration (A), cells mL⁻¹ and PO₄³⁻ (B), SiO₄⁴⁻ (C), and NO₃⁻ (D) concentrations (μmol L⁻¹) along the exponential growth phase in the enriched medium with water movement.

On the role of enriched medium together with water movement. Despite high cell concentration ($\approx 750,000$ cells mL⁻¹; Fig. 4A), PO₄³⁻ and SiO₄⁴⁻ concentrations were consistently high, around 60 μmol L⁻¹ (Fig. 4B) and ≈ 200 μmol L⁻¹ (Fig. 4C), respectively. At the same cell concentration, NO₃⁻ was still very high (700 μmol L⁻¹, Fig. 4D) in the range of the concentration reported in the f/2 medium (Figs 1D, 3D). Enriching the medium prevents limitation of growth by nutrient depletion and culture reached the stationary phase independently of macronutrients' limitation. Therefore, the population death phase is not explained, and different hypotheses might be advanced: from high level of intracellular reactive oxygen species produced during cell senescence, to external causes such as virus attacks or a death induced by a high concentration of toxic molecules (extracellular self DNA or sugars) in the medium such as observed in recent studies on higher plants or yeasts^{55,56}. This last hypothesis well fit with the very high growth rate reached by cells, inducing a high excretion rate of products, and requires further studies.

The enriched medium induces a further and significant enhancement of growth rate ($p < 0.01$) compared to the original f/2 medium. Assumption is that the probability that cells encounter soluble nutrients to acquire is strongly increasing by doubling their concentrations together with water movement. The most relevant changes in nutrient acquisition in function of growth rate are noteworthy for PO₄³⁻ and SiO₄⁴⁻ (Table 4). Conversely, similar NO₃⁻ uptake between enriched and conventional medium indicates that significant growth rate variations do not modulate NO₃⁻ uptake suggesting and reinforcing the previous discussion on nitrogen acquisition under other N forms (urea, amino acids)⁴⁶. Indeed, similar cellular NO₃⁻ uptake is reported in the different growth rate classes (Table 4). PO₄³⁻ and SiO₄⁴⁻ uptake strongly decreased from low to moderate-high growth rate classes ($p < 0.01$, Table 4). As consequence, N:P and N:Si ratios increased from low to moderate/high growth rate ($p < 0.05$, Table 4).

Comparing the three culture conditions, PO₄³⁻ uptake is enhanced by water movement and further in enriched medium, such as growth rate, confirming the expected higher P requirement by cells with increasing growth rate, as also stated by the GRH. The three classes of P-containing compounds were significantly higher than in cells grown in the original f/2 medium ($p < 0.05$), although P allocation in cells varied with growth rate (Table 4). The P-RNA content surprisingly decreased with increasing growth rate (Table 4). Interestingly, in each growth rate class relationship between growth rate and P-RNA (phosphorus attributed to RNA) was significant ($p < 0.001$, Table 5) with the slope of the regression showing a decreasing trend from low to very high growth rate, except for the highest growth rate class (Table 5). This result is in agreement with the GRH^{14,57} and confirms growth rate as driver of the biochemical machinery of cells to run the genetic expression required by cells for cell cycle progression and division. The lack of robustness of the correlation between growth rate and P-RNA when pooling together all data from the different classes confirms that the complicated relationship between RNA content, P uptake and growth rate in photosynthetic organisms¹³. The intracellular P pool dynamics¹³ such as variations of P allocation into the different intracellular compounds (RNA, DNA, stored, etc.)¹³ along with growth phase might be the reasons for such complexity. For instance, in the low growth rate class – corresponding to cells in lag or post-exponential phase – the higher P uptake compared to the other classes might indicate an enhancement of the stored P pool or of the P used for repair activity.

Growth rate	N	P	Si	N:P	N:Si	P:Si	P-RNA	P-storage	P-no_storage
-4.70 d ⁻¹ to -0.01 d ⁻¹ (n = 42)	2.89 ± 0.40	0.67 ± 0.07	0.36 ± 0.05	4.29 ± 0.53	8.01 ± 1.11	1.87 ± 0.22	4.14 ± 0.32	3.34 ± 0.20	13.36 ± 1.00
0.00 d ⁻¹ to 0.70 d ⁻¹ (n = 24)	0.71 ± 0.10	0.24 ± 0.03	0.25 ± 0.05	2.97 ± 0.39	2.77 ± 0.46	0.93 ± 0.14	0.15 ± 0.05	1.46 ± 0.41	5.84 ± 1.73
0.71 d ⁻¹ to 0.90 d ⁻¹ (n = 32)	0.59 ± 0.08	0.05 ± 0.004	0.09 ± 0.01	11.9 ± 1.32	6.03 ± 0.65	0.50 ± 0.04	0.09 ± 0.01	0.28 ± 0.03	1.12 ± 0.12
0.91 d ⁻¹ to 1.20 d ⁻¹ (n = 26)	0.82 ± 0.10	0.07 ± 0.01	0.10 ± 0.01	10.65 ± 1.85	7.56 ± 0.86	0.71 ± 0.11	0.07 ± 0.02	0.42 ± 0.10	1.68 ± 0.51
1.21 d ⁻¹ to 1.90 d ⁻¹ (n = 31)	0.72 ± 0.3	0.09 ± 0.02	0.04 ± 0.006	7.81 ± 1.50	16.7 ± 2.56	2.13 ± 0.26	0.04 ± 0.02	0.56 ± 0.30	2.24 ± 1.07

Table 4. Nutrient uptake (pmol cells⁻¹ d⁻¹) and ratios between N:P, N:Si and P:Si uptaken by cells grown in an enriched medium with water movement. P-RNA (pg) is for P content allocation in RNA (using P = 0.091 g dry g RNA⁻¹)¹². P-storage (pg) is for P content allocation in reserve compounds (inorganic phosphate) and P-no storage (pg) is for P content allocation in other phosphorus-containing compounds. n is the number of data for each class of growth rate.

Growth rate	Correlation
-4.70 d ⁻¹ to -0.01 d ⁻¹	$y = -7.21x - 3.57 R^2 = 0.54$ (p < 0.001)
0.00 d ⁻¹ to 0.70 d ⁻¹	$y = 0.62x + 0.027 R^2 = 0.57$ (p < 0.001)
0.71 d ⁻¹ to 0.90 d ⁻¹	$y = 0.36x - 0.028 R^2 = 0.89$ (p < 0.001)
0.91 d ⁻¹ to 1.20 d ⁻¹	$y = 0.12x - 0.004 R^2 = 0.41$ (p < 0.001)
1.21 d ⁻¹ to 1.90 d ⁻¹	$y = 0.29x - 0.26 R^2 = 0.79$ (p < 0.001)

Table 5. Enriched medium. Spearman correlation between P-RNA (y, pg) and growth rate (x, d⁻¹) for each of the growth rate class.

Conclusions

This study assessed the effects of physical motion (water movement vs. air bubbling) and nutrient concentrations on the growth rate by the coastal centric diatom *Skeletonema marinoi*. Water movement enhanced growth rate and modulated nutrient acquisition rate. Enriched medium, compared to conventional f/2 medium, further enhanced growth rate and biomass harvesting. Results of this study contribute to enhancing diatom's biomass productivity, reducing its cost, as it is required in the blue biotechnology context.

References

- Barra, L., Chandrasekaran, R., Corato, F. & Brunet, C. The challenge of ecophysiological biodiversity for biotechnological applications of marine microalgae. *Marine Drugs* **12**, 1641–1675 (2014).
- Qin, S., Lin, H. & Jiang, P. Advances in genetic engineering of marine algae. *Biotechnology Advances* **30**, 1602–1613 (2012).
- Orefice, I. et al. Light-induced changes in the photosynthetic physiology and biochemistry in the diatom *Skeletonema marinoi*. *Algal Research* **17**, 1–13 (2016).
- Smerilli, A. et al. Photoprotective and antioxidant responses to light spectrum and intensity variations in the coastal diatom *Skeletonema marinoi*. *Environmental Microbiology* **19**, 611–627 (2017).
- Raven, J. A. & Geider, R. J. Temperature and algal growth. *New Phytologist* **110**, 441–461 (1988).
- Litchman, E. & Klausmeier, C. A. Trait-based community ecology of phytoplankton. *Annual Review of Ecology, Evolution, and Systematics* **39**, 615–639 (2008).
- Grossman, A. Nutrient Acquisition: The generation of bioactive vitamin B₁₂ by microalgae. *Current Biology* **26**, R319–R321 (2016).
- Martin-Jezequel, V., Hildebrand, M. & Brzezinski, M. A. Silicon metabolism in diatoms: implications for growth. *Journal of Phycology* **36**, 821–840 (2000).
- Shrestha, R. P. & Hildebrand, M. Evidence for a regulatory role of diatom silicon transporters in cellular silicon responses. *Eukaryotic Cell* **14**, 29–40 (2015).
- Lewin, J. C. Silicon metabolism in diatoms. III. Respiration and silicon uptake in *Navicula pelliculosa*. *The Journal of General Physiology* **39**, 1–10 (1955).
- Norici, A., Bazzoni, A. M., Pugnetti, A., Raven, J. A. & Giordano, M. Impact of irradiance on the C allocation in the coastal marine diatom *Skeletonema marinoi* Sarno and Zingone*. *Plant Cell. Environment* **34**, 1666–1677 (2011).
- Geider, R. J. & La Roche, J. Redfield revisited: variability of C:N:P in marine microalgae and its biochemical basis. *European Journal of Phycology* **37**, 1–17 (2002).
- Raven, J. A. The evolution of autotrophy in relation to phosphorus requirements. *Journal of Experimental Botany* **64**, 4023–4046 (2013).
- Sterner, R. W. & Elser, J. J. Ecological stoichiometry: the biology of elements from molecules to the biosphere. Princeton Univ. Press, Princeton (2002).
- Eixler, S., Karsten, U. & Selig, U. Phosphorus storage in *Chlorella vulgaris* (Trebouxiophyceae, Chlorophyta) cells and its dependence on phosphate supply. *Phycologia* **45**, 53–60 (2006).
- Ho, T. Y. et al. The elemental composition of some marine phytoplankton. *Journal of Phycology* **39**, 1145–1159 (2003).
- Litchman, E., Klausmeier, C. A., Schofield, O. M. & Falkowski, P. G. The role of functional traits and trade-offs in structuring phytoplankton communities: scaling from cellular to ecosystem level. *Ecology Letters* **10**, 1170–1181 (2007).
- Berges, J. A., Varela, D. E. & Harrison, P. J. Effects of temperature on growth rate, cell composition and nitrogen metabolism in the marine diatom *Thalassiosira pseudonana* (Bacillariophyceae). *Marine Ecology Progress Series* **225**, 139–146 (2002).
- Klausmeier, C. A., Litchman, E. & Levin, S. A. Phytoplankton growth and stoichiometry under multiple nutrient limitation. *Limnology and Oceanography* **49**, 1463–1470 (2004).
- Sanz-Luque, E., Chamizo-Ampudia, A., Llamas, A., Galvan, A. & Fernandez, E. Understanding nitrate assimilation and its regulation in microalgae. *Frontiers in Plant Science* **6**, 899, <https://doi.org/10.3389/fpls.2015.00899> (2015).
- Thrane, J. E. et al. Spectrophotometric analysis of pigments: a critical assessment of a high-throughput method for analysis of algal pigment mixtures by spectral deconvolution. *PLoS ONE* **10**, e0137645, <https://doi.org/10.1371/journal.pone.0137645> (2015).

22. Flynn, K. J. *et al.* Is the growth rate hypothesis applicable to microalgae? *Journal of Phycology* **46**, 1–12 (2010).
23. Wang, C. & Lan, C. Q. Effects of shear stress on microalgae - A review. *Biotechnology Advances* **36**, 986–1002, <https://doi.org/10.1016/j.biotechadv.2018.03.001> (2018).
24. Thomas, W. H. & Gibson, C. H. Effects of small-scale turbulence on microalgae. *Journal of Applied Phycology* **2**, 71–77 (1990).
25. Ogbonna, J. C., Yada, H. & Tanaka, H. Effect of cell movement by random mixing between the surface and bottom of photobioreactors on algal productivity. *Journal of fermentation and bioengineering* **79**, 152–157 (1995).
26. Richmond, A. Principles for attaining maximal microalgal productivity in photobioreactors: an overview. In *Asian Pacific Phycology in the 21st Century: Prospects and Challenges*, 33–37, Springer, Dordrecht (2004).
27. Taraldsvik, M. & Myklestad, S. M. The effect of pH on growth rate, biochemical composition and extracellular carbohydrate production of the marine diatom *Skeletonema costatum*. *European Journal of Phycology* **35**, 189–194 (2000).
28. Bartley, M. L., Boeing, W. J., Dungan, B. N., Holguin, F. O. & Schaub, T. pH effects on growth and lipid accumulation of the biofuel microalgae *Nannochloropsis salina* and invading organisms. *Journal of applied phycology* **26**, 1431–1437 (2014).
29. Chalmers, J. J. Mixing, aeration and cell damage. 30+ years later: what we learned, how it affected the cell culture industry and what we would like to know more about. *Current Opinion in Chemical Engineering* **10**, 94–102, <https://doi.org/10.1016/j.coche.2015.09.005> (2015).
30. Chandrasekaran, R. *et al.* Light modulation of biomass and macromolecular composition of the diatom *Skeletonema marinoi*. *Journal of Biotechnology* **192**, 114–122 (2014).
31. Brown, M. R., Jeffrey, S. W., Volkman, J. K. & Dunstan, G. A. Nutritional properties of microalgae for mariculture. *Aquaculture* **151**, 315–331 (1997).
32. Liao, I. C., Su, H. M. & Lin, J. H. Larval foods for Penaeid prawns. Crustacean. *Aquaculture* **1**, 43–68 (1983).
33. Su, H. M., Lei, C. H. & Liao, I. C. Effect of temperature, illumination and salinity on the growth rates of *Skeletonema costatum*. *Journal of fisheries Society Taiwan* **17**, 213–222 (1990).
34. Coutteau, P. & Sorgeloos, P. The use of algal substitutes and the requirement for live algae in the hatchery and nursery rearing of bivalve molluscs: An international survey. *Journal of Shellfish Research* **11**, 467–476 (1992).
35. Kourtchenko, O., Rajala, T. & Godhe, A. Growth of a common planktonic diatom quantified using solid medium culturing. *Scientific Reports* **8**, 9757 (2018).
36. Brunet, C. *et al.* Spectral radiation dependent photoprotective mechanism in diatom *Pseudo-nitzschia multistriata*. *PLoS One* **9**, e87015, <https://doi.org/10.1371/journal.pone.0087015> (2014).
37. Guillard, R. R. L. & Ryther, J. H. Studies of marine planktonic diatoms. I. *Cyclotella nana* Husted, and *Detonula confervacea* (Cleve) Gran. *Canadian Journal of Microbiology* **8**, 229–239 (1962).
38. Grasshoff, K., Kremling, K. & Ehrhardt, M. *Methods of Seawater Analysis*. Wiley, New York (2009).
39. Barra, L., Ruggiero, M. V., Sarno, D., Montresor, M. & Kooistra, W. C. F. Strengths and weaknesses of microarray approaches to detect *Pseudo-nitzschia* species in the field. *Environmental Science and Pollution Research* **20**, 6705–6718 (2013).
40. Hammer, Ø., Harper, D. A. T. & Ryan, P. D. PAST: Paleontological Statistics Software Package for Education and Data Analysis. *Palaeontologia Electronica* **4**, 9 (2001).
41. Guillard, R. R. L. Culture of phytoplankton for feeding marine invertebrates, in: Smith, W. L. & M. H. Chanley (Eds.), *Culture of marine invertebrate animals*, Plenum Press, New York, 26–60 (1975).
42. Dell'Aquila, G. *et al.* Nutrient consumption and chain tuning in diatoms exposed to storm-like turbulence. *Scientific Reports* **7**, 1828, <https://doi.org/10.1038/s41598-017-02084-6> (2017).
43. Nakamura, H. *et al.* Utilization of ammonium by the hydrocarbon-producing microalga, *Botryococcus braunii* Showa. *Algal Research* **2**, 445–451 (2017).
44. Pujol-Pay, M., Conan, P. & Raimbault, P. Underestimation of particulate nitrogen concentrations in open ocean by the use of GF/F filters. *Comptes Rendus de l'Académie des Sciences* **324**, 401–407 (1997).
45. Jones, D. T. *et al.* Solvent production and morphological changes in *Clostridium acetobutylicum*. *Applied and Environmental Microbiology* **43**, 1434–1439 (1982).
46. Flynn, K. J. & Butler, I. Nitrogen sources for the growth of marine microalgae: role of dissolved free amino acids. *Marine Ecology Progress Series* **34**, 281–304 (1986).
47. Hellebust, J. A. & Ahmad, I. Regulation of Nitrogen Assimilation in Green Microalgae. *Biological Oceanography* **6**, 241–255 (1989).
48. Franco, A. R., Cárdenas, J. & Fernández, E. Regulation by ammonium of nitrate and nitrite assimilation in *Chlamydomonas reinhardtii*. *Biochimica et Biophysica Acta* **951**, 98–103 (1988).
49. Fernandez, E. & Galvan, A. Nitrate assimilation in *Chlamydomonas*. *Eukaryotic Cell* **7**, 555–559 (2008).
50. Barton, A. D., Ward, B. A., Williams, R. G. & Follows, M. J. The impact of fine-scale turbulence on phytoplankton community structure. *Limnology and Oceanography: Fluids and Environments* **4**, 34–49 (2014).
51. Amato, A. *et al.* Marine diatoms change their gene expression profile when exposed to microscale turbulence under nutrient replete conditions. *Scientific Reports* **7**, 3826, <https://doi.org/10.1038/s41598-017-03741-6> (2017).
52. Peters, F., Arin, L., Marrasé, C., Berdalet, E. & Sala, M. M. Effects of small-scale turbulence on the growth of two diatoms of different size in a phosphorus-limited medium. *Journal of Marine Systems* **61**, 134–148 (2005).
53. Spitzer, S. An analysis of diatom growth rate and the implications for the biodiesel industry. *Occam's Razor* **5**, <https://cedar.wvu.edu/orwvu/vol5/iss1/6> (2015).
54. Levitan, O., Dinamarca, J., Hochman, G. & Falkowski, P. G. Diatoms: a fossil fuel of the future. *Trends in Biotechnology* **3**, 117–124 (2014).
55. Mazzoleni, S. *et al.* Inhibitory and toxic effects of extracellular self-DNA in litter: a mechanism for negative plant-soil feedbacks? *New Phytologist* **205**, 1195–1210 (2015).
56. de Alteriis, E., Carteni, F., Parascandola, P., Serpa, J. & Mazzoleni, S. Revisiting the Crabtree/Warburg effect in a dynamic perspective: a fitness advantage against sugar-induced cell death. *Cell Cycle* **17**, 688–701 (2018).
57. Sterner, R. W. *et al.* Scale-dependent carbon: nitrogen: phosphorus seston stoichiometry in marine and freshwaters. *Limnology and Oceanography* **53**, 1169–1180 (2008).

Acknowledgements

Ida Orefice was granted by a post-doctoral fellowship in the framework of the SZN Research project MARCAN. Clementina Sansone was granted by a post-doctoral EMBRIC H2020 fellowship. Raghu Chandrasekaran and Arianna Smerilli have been supported by a Stazione Zoologica Anton Dohrn PhD fellowship. The authors acknowledge the reviewers for their comments.

Author Contributions

F.C. and C.B. designed the light system and experimental setup adopted during the experiments; C.B. designed the study; I.O., A.S., R.C., F.C. and C.B. performed the experiments and the analysis. M.M. and C.B. built up the dataset and made the statistical analysis. M.M., I.O., C.S. and C.B. interpreted and discussed the results; all authors drafted the manuscript; all authors approved final version of manuscript.

Additional Information

Competing Interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2019