Plant, Cell & Environment

Plant, Cell and Environment (2017) **40**, 227–236 doi: 10.1111/pce.12858

Original Article

The use of NH_4^+ rather than NO_3^- affects cell stoichiometry, C allocation, photosynthesis and growth in the cyanobacterium *Synechococcus* sp. UTEX LB 2380, only when energy is limiting

Zuoxi Ruan[1,2] & Mario Giordano[2,3,4]

[1]*Marine Biology Institute, Science Center, Shantou University, Shantou, Guangdong 515063, China,* [2]*Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Ancona 60131, Italy,* [3]*Institute of Microbiology ASCR, Algatech, Trebon, Czech Republic and* [4]*National Research Council, Institute of Marine Science, Venezia, Italy*

ABSTRACT

The assimilation of N-NO_3^- requires more energy than that of N-NH_4^+. This becomes relevant when energy is limiting and may impinge differently on cell energy budget depending on depth, time of the day and season. We hypothesize that N-limited and energy-limited cells of the oceanic cyanobacterium *Synechococcus* sp. differ in their response to the N source with respect to growth, elemental stoichiometry and carbon allocation. Under N limitation, cells retained almost absolute homeostasis of elemental and organic composition, and the use of NH_4^+ did not stimulate growth. When energy was limiting, however, *Synechococcus* grew faster in NH_4^+ than in NO_3^- and had higher C (20%), N (38%) and S (30%) cell quotas. Furthermore, more C was allocated to protein, whereas the carbohydrate and lipid pool size did not change appreciably. Energy limitation also led to a higher photosynthetic rate relative to N limitation. We interpret these results as an indication that, under energy limitation, the use of the least expensive N source allowed a spillover of the energy saved from N assimilation to the assimilation of other nutrients. The change in elemental stoichiometry influenced C allocation, inducing an increase in cell protein, which resulted in a stimulation of photosynthesis and growth.

Key-words: carbon allocation; cyanobacteria; elemental stoichiometry; energy; FTIR; nitrogen.

INTRODUCTION

Nitrate and ammonium are the most common forms of combined inorganic nitrogen in the ocean (Raven & Giordano 2016), where N typically controls primary productivity (Falkowski 1997). Phytoplankton, with some exceptions, can utilize both NO_3 and NH_4^+ as the N source (Moore *et al.* 2002; Raven & Giordano 2016). The cost of N-NO_3^- and N-NH_4^+ assimilation is substantially different, because of the fact that the incorporation of N into the organic matter occurs at the lowest oxidation number (−3, that of NH_4^+); therefore, the N (+6) of NO_3^- requires a larger amount of reducing power for its assimilation into amino acids. The assimilation of NH_4^+ into amino acids occurs through the glutamine synthetase/glutamate oxoglutarate aminotransferase system (GS/GOGAT), which requires about three ATP equivalents; the reduction of NO_3^- to NH_4^+ requires eight additional ATP equivalents (Cullimore & Sims 1981; Guerrero *et al.* 1981; Vega *et al.* 1987; Turpin 1991). On average, a typical phytoplankton cell has a C:N:P:S cell stoichiometry of 124:16:1:1.3 (Giordano 2013 and refereces therein). Consequently, at least 13% more energy is needed to produce the same amount of biomass, if NO_3^- rather than NH_4^+ is used. In addition, when the capital cost of the machinery needed for NO_3^- assimilation is also taken into account (e.g. synthesis of nitrate transporters, nitrate reductase and nitrite reductase), the energetic advantage of using ammonium increases further (Huppe & Turpin 1994). The lower cost of NH_4^+assimilation, however, becomes relevant only when energy is limiting. The fact that this is not always considered may explain the somewhat erratic results reported in the literature for growth on NH_4^+ or NO_3^- in the lab (Giordano 1997; Kudo & Harrison 1997; Sinclair *et al.* 2009; Collier *et al.* 2012). In the sea, phytoplankton is often exposed to low irradiances. For instance, light limitation can occur during blooms (Balch *et al.* 1991), in estuarine environment with turbid waters (Kruk *et al.* 2015), at higher latitude during winter (Mitchell *et al.* 1991; Strom *et al.* 2010), in the course of vertical migration or even in surface seawater due to cloud coverage or low solar elevation (Hopkinson & Barbeau 2008; Waite & Mueter 2013).

It should be considered that algae can sustain a similar growth rate even if the N cell quota is different. This may occur when cells modify their N use efficiency. For instance, this happens when cells activate CO_2 concentrating mechanisms (Beardall *et al.* 1982, 1991; Giordano *et al.* 2005; Raven & Beardall 2016) or are transferred to elevated CO_2, conditions under which the amount of Rubisco, one of the main cell repositories of N, decreases (Beardall & Giordano 2002; Raven *et al.* 2011). If the C:N ratio is altered, also C partitioning among the various organic pools may be affected. Most of the N in a cell is allocated to protein and amino acids, with a

Correspondence: M. Giordano. Tel.: +39 071 220 4652; e-mail: m.giordano@univpm.it

C:N ratio of 3.8, and to a smaller extent to nucleic acid, with a C:N ratio of 2.25 (see for instance Norici *et al.* 2011 and references therein). The C in excess to this ratio must thus be allocated to pools that do not contain N, such as carbohydrates and lipids. These pools are necessary for the function and the structures of a cell; if, however, the C in excess to N is more than that required for the functional and structural carbohydrates and lipids, then storage pools are accumulated. The storage pools, depending on conditions and genotypes, can be constituted by lipids or carbohydrates or both (Palmucci *et al.* 2011; Palmucci & Giordano 2012). Changes in the allocation of C to the various pools have energetic repercussions, because the amount of energy required to fix C into protein, lipids and carbohydrates is different (Raven 1982; Palmucci *et al.* 2011). Therefore, the cost of assimilatory processes, such as N assimilation, may constrain C allocation by altering the overall energy available for biosynthetic pathways. When light is limiting, choices must be made on where the energy has to be invested, and the assimilation of either NO_3^- or NH_4^+, which have different energy requirements, becomes relevant. Conversely, the chemical source of N may be of little relevance if energy is in large supply (i.e. light is saturating or supersaturating).

We chose the cyanobacterium *Synechococcus* as our experimental organism; we did so because *Synechococcus* is a major contributor to oceanic primary production (Agawin *et al.* 1998). Together with *Prochlorococcus*, it is probably the most abundant phototrophic oceanic organisms (Partensky *et al.* 1999; Scanlan 2003), in terms of cell abundance: it has been estimated that it can constitute up to 60% of phytoplankton, in certain areas (Worden *et al.* 2004). *Synechococcus* is ubiquitously distributed, although it is less abundant in polar waters, and may encounter various inorganic N species and be exposed to different light regimes (Vincent 2002; Scanlan 2003, 2012). Furthermore, we chose a cyanobacterium because organisms of this group may be especially sensitive to changes in light availability because of the higher energy costs of pigment synthesis (1.5 to 3.7-fold that of eukaryotic algae) and lower specific absorption coefficient of harvesting complexes, as compared with eukaryotic algae (Raven 1984a).

Based on all the previous texts, we hypothesize that, when light (=energy) is limiting, the chemical form in which N is assimilated influences the amount of energy available for biosynthesis and may therefore affect C allocation and, ultimately, growth.

MATERIALS AND METHODS

Culture conditions

The unicellular marine non-diazotrophic cyanobacterium *Synechococcus* UTEX LB 2380 was cultured at a temperature of 25 °C, with a photon flux density (PFD) of 100 μmol photons·m^{-2}·s^{-1} and a 12:12 light-dark photoperiod, in 250 mL Erlenmeyer flasks containing 200 mL of algal suspension. To minimize cell sinking and walling, the flasks were manually shaken twice a day, during the light period.

The growth medium was the Artificial Multipurpose Complement for the Nutrition of Algae (AMCONA) medium (Fanesi *et al.* 2014). In its standard formulation, this medium contains 550 μM nitrate, which, in our culture conditions, did not limit *Synechococcus* growth (Fig. 1). Cells were also cultured in AMCONA medium containing 550 μM ammonium, which also did not limit growth. Growth limitation was obtained by decreasing nitrate/ammonium concentration to 22 μM (Fig. 1). Cultures at 2000 μM either NO_3^- or NH_4^+ were also used in preliminary experiments, in order to assess limitation (Fig. 1). For the experiments, semi-continuous cultures were used, which were diluted at the following rates: 0.40 d^{-1} (22 μM NO_3^-), 0.35 d^{-1} (22 μM NH_4^+), 0.60 d^{-1} (550 μM NO_3^-) and 0.67 d^{-1} (550 μM NH_4^+). After dilution, the number of cells in an mL of culture was around 2–3·10^6 cells, in all cultures. The dilution rates were chosen so that they corresponded to the maximum growth rates measured in batch cultures after the cells were acclimated to each growth condition for at least eight generations. After the semicontinuous cultures were established, the cells were allowed to acclimate for at least further 10 generations (or until steady state growth was attained), prior to being used for the experiments. Measurements were carried out on samples collected between hours 3 and 4 of the light period.

Determination of cell number and cell volume

Cell number and cell volume were determined daily with a CASY TT cell counter (Innovatis AG, Reutlingen, Germany). These measurements were carried out 4 h after the onset of the light period. A culture aliquot of 100 μL was diluted in 10 mL of electrolyte solution CASY®ton (Innovatis AG, Reutlingen, Germany), which had been previously filter-sterilized through a 0.2 μM mixed cellulose ester filter

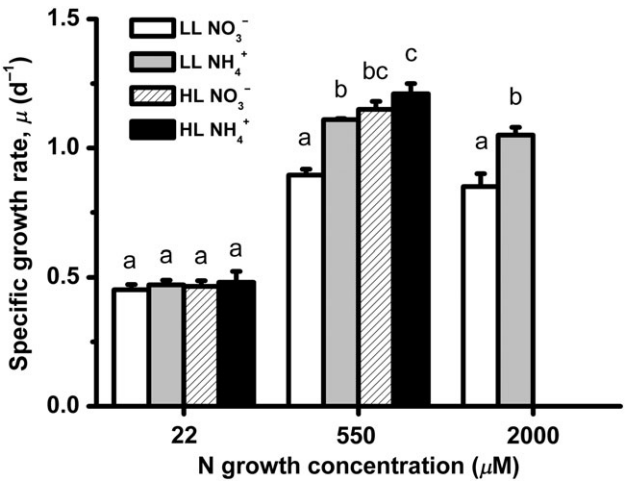

Figure 1. Growth rate of *Synechococcus* sp. UTEX LB 2380 acclimated to 22, 550 or 2000 μM either NO_3^- or NH_4^+, under a photon flux density of either 100 μmol photons·m^{-2}·s^{-1} (LL) or 200 μmol photons·m^{-2}·s^{-1} (HL). Different letters in the superscript denote significantly different means ($P < 0.05$). The error bars indicate the standard deviations ($n \geq 3$).

(Schleicher & Schuell BioScience GmbH, Dassel, Germany). The background particles in the range 1.6–4.0 μm were always determined in fresh cell-free medium diluted with electrolyte solution as the culture samples; these background values were used to correct the cell counts.

Measurement of dry weight and estimation of biomass productivity

For dry weight measurements, about $0.5–1 \times 10^9$ cells were collected by centrifugation with a MPW351 bench centrifuge (MPW med. Instrument, Warsaw, Poland), at 3400 g for 10 min. The cells were washed twice with a 0.5 M ammonium formate solution (isosmotic to the growth medium) (Kröcher *et al.* 2009). This allowed to get rid of medium salts. The cells were then spun down in pre-weighed 1.5 mL Eppendorf tubes and dried at 80 °C for at least 2 d or until the weight was stable. The dry weight was used to estimate biomass productivity according to the following equation:

$$\text{Biomass productivity} = \mu \cdot \text{DW}$$

where μ is the specific growth rate (d^{-1}) and DW is the cellular dry weight ($pg \cdot d^{-1}$).

Total protein determination

For protein determination, about $0.5–2.0 \times 10^7$ cells were harvested by centrifugation (13 700 g, 5 min). The amount of protein was determined according to Lowry *et al.* (1951), as modified by Peterson (1977). Bovine serum albumin was used as standard.

Determination C, N and S cell quotas

The cell quotas of C, N and S were determined with an elemental analyser (EA1108, Carlo Erba Instruments, Milan, Italy). Samples for C, N and S analyses were prepared as for the dry weight determinations. About 1.5–5 mg of dry sample were wrapped in a tin capsule, together with a small amount of V_2O_5, which facilitates the complete oxidation of the sample. Sulfanilamide (C 41.84%, N 16.27%, S 18.62% W/W) was used as a standard; a standard curve with at least eight concentration points (1 to 5 mg sulfanilamide) was constructed; two standards were always measured at the beginning of each run, then a standard was measured every two to three samples. The amount of C, N and S was derived from the interpolation of the experimental data into the standard curve.

The macronutrient assimilation rate was calculated by multiplying the cell quotas by the specific growth rate.

The elemental stoichiometry was expressed as molar ratios.

Determination of organic cell composition

Organic cell composition was determined by Fourier transform infrared spectroscopy (FTIR). For FTIR analyses, about $1–2 \times 10^8$ cells were harvested. The cells were initially treated as for the dry weight determination. The pellet was then resuspended in 200 μL of 0.5 M ammonium formate; 50 μL of this cell suspension were deposited on a monocrystalline silicon window (Crystran Ltd., Dorset, U.K.). A 50 μL aliquot of ammonium formate solution was used as blank. Samples and blanks were desiccated in an oven at 80 °C for at least 4 h. They were then analysed with a Tensor 27 FTIR spectrometer (Brüker Optics, Ettlingen, Germany). Spectral acquisition and analyses were conducted as described in Domenighini & Giordano (2009) and Marchetti *et al.* (2010). Bands were assigned to the various pools according to Giordano *et al.* (2001). Semiquantification of the carbohydrate and lipid pools was carried out according to Palmucci *et al.* (2011). The carbohydrate and lipid content were then expressed relative to the lowest value measured, to which a value of 1 was assigned. In this data set, the lowest value was that of lipids for cells grown on 550 μM NH_4^+.

The production rate of the organic pools was calculated by multiplying the cell pool size by the specific growth rate.

Photosynthetic O_2 evolution

Photosynthetic O_2 evolution was measured in a Chlorolab 2 system (Hansatech, Norfolk, UK), according to the procedure described by Ratti *et al.* (2007), at saturating dissolved inorganic C and at growth irradiance ($100\ \mu mol \cdot m^{-2} \cdot s^{-1}$)

Cells ($3–6 \times 10^7$) were collected by centrifugation (3400 g, 10 min), resuspended in 2 mL of fresh AMCONA medium and transferred into the O_2 electrode chamber. The O_2 evolution was monitored with a 1 s frequency, using the Labview 2008 software (National instruments, Milano, Italy). Light was provided by an LS2 100 W tungsten halogen light source (Hansatech Instrument, Norfolk, UK).

Statistics

All data were acquired from at least three distinct cultures. The results were expressed as means and standard deviation. The significance of variance was assessed by two-tailed *t*-test or by two-way ANOVA analysis. In the two-way ANOVA, the homogeneity of variance was checked by the Cochran's test before analysis; if the outcome of this test was not satisfactory, appropriate models were applied to transform the original data until variance homogeneity was reached. The significance level was always set at 95%. All statistical tests were performed with the Origin 7.0 SR0 software (OriginLab Corporation, Northampton, USA).

RESULTS

Growth rates

At high N concentration (550 μM) and at 100 μmol photons$\cdot m^{-2} \cdot s^{-1}$, cells grown in the presence of NH_4^+ as the sole N source grew faster than those grown in NO_3^-; an increase of neither NO_3^- nor NH_4^+ concentration elicited a stimulation of growth rate (Fig. 1). At the same N concentration, growth at 200 μmol photons$\cdot m^{-2} \cdot s^{-1}$ caused a 29 and 9% increase of specific growth rate (μ) for NO_3^--grown and

NH_4^+-grown cells, respectively (Fig. 1). This confirms that light and not N concentration limited growth in our high N cultures. When light was increased, the difference between NO_3^--grown and NH_4^+-grown cells became statistically non-significant (Fig. 1). When cells were grown at an N concentration of 22 μM, PFD had no influence on the growth rate, and growth was N-limited (Fig. 1). In these N-limited cultures, the chemical form of N had no effect on μ (Fig. 1).

Cell dry weight and cell volume

Cells were about 47% (NO_3^-) and 65% (NH_4^+) lighter in N-limited (22 μM N) than in light (energy)-limited (550 μM N) conditions (Table 1). The dry weight of N-limited cells was unaffected by the N-source (Table 1). This was not the case for the energy-limited cultures: in this case, cells grown on NH_4^+ were about 26% heavier than their NO_3^--grown counterparts.

In general, N-limited cells were always smaller than energy-limited cells; this was especially obvious when N was supplied as NH_4^+ (Table 1).

The volume of N-limited cells was slightly larger (7%) in the presence of NO_3^- than of NH_4^+. The opposite trend was observed when cells were energy-limited; in this case, the volume was about 18% smaller for cells grown in NO_3^- (Table 1).

Macronutrients cell quotas, cell stoichiometry and assimilation rates

The C quota was not significantly different in N-limited and energy-limited cells, when NO_3^- was the N source. However, N-limited cells contained 24% less C than energy-limited cells, when N was provided as NH_4^+.

N cell quotas of N-limited cells were 30% (NO_3^-) and 60% (NH_4^+) lower than those of energy-limited cells.

Sulphur cell quotas were 22% (NO_3^-) and 33% (NH_4^+) lower in N-limited than in energy-limited cells.

The P quotas were not influenced by the N availability.

When the cells were N-limited, the N source had no statistically significant effect on the C, N, S and P cell quotas. Under energy-limitation only, the P quotas were unaffected by the N source, whereas the cell quotas of C, N and S were about 17, 28 and 23% lower in NO_3^- than in NH_4^+, respectively (Table 1).

The stoichiometrical relationships of C, N and P (i.e. C/N and N/P) were significantly influenced by the factor limiting growth (energy or N). A lower C/N ratio was observed when energy was limiting; the opposite was true for the N/P ratio. The C/N ratio was also influenced by the N chemical form (Fig. 2): it was 10% lower in NO_3^- than in NH_4^+ when N was limiting, but an opposite trend was observed when energy was limiting; in this case, the C/N ratio was 11% higher in NO_3^- than in NH_4^+. It is noteworthy that, in the presence of NO_3^-, the N/S ratio was very similar in both N-limited and energy-limited cells, but, in NH_4^+, the N/S ratio was much lower (40%) in N-limited than in energy-limited cells. The N/S ratio was appreciably higher in NO_3^- than in NH_4^+ grown cells, when N was limiting; such effect of the N chemical form was not visible under energy limitation.

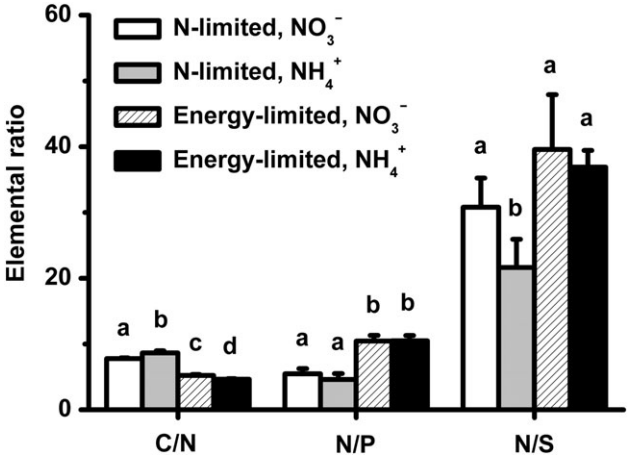

Figure 2. Stoichiometry (C/N, N/P, N/S molar ratios) of macronutrients in cells of *Synechococcus* sp. UTEX LB 2380, under either N or energy limitation. Different letters in the superscript denote significantly different means ($P < 0.05$). The error bars indicate the standard deviations ($n \geq 3$).

Table 1. Dry weight, cell volume, macronutrients quotas and organic pool size of *Synechococcus* sp. UTEX LB 2380 cells under N or energy limitation. Means and standard deviation (in parentheses) were calculated from at least three independent cultures. Different letters in the superscript denote significantly different means within groups of measurements ($P < 0.05$)

					Macronutrients				Organic pools		
		Growth rate	Dry weight	Cell volume	C	N	S	P	Protein	Carbohydrate	Lipid
Limitation	N form	d^{-1}	$pg \cdot cell^{-1}$	μm^3	$fg \cdot cell^{-1}$	$fg \cdot cell^{-1}$	fg $cell^{-1}$	$fg \cdot cell^{-1}$	$fg \cdot cell^{-1}$	r.u.	r.u.
Nitrogen	NO_3^-	0.45^{a}	1.24^{a}	5.37^{a}	723^{ab}	109^{a}	8.2^{a}	44.8^{a}	762^{a}	1.35^{a}	2.55^{a}
		(0.02)	(0.13)	(0.12)	(96)	(16)	(0.4)	(4.2)	(138)	(0.05)	(0.10)
	NH_4^+	0.47^{a}	1.02^{a}	5.02^{b}	639^{a}	86^{a}	9.1^{a}	41.5^{a}	844^{a}	1.54^{a}	2.84^{a}
		(0.02)	(0.20)	(0.08)	(117)	(17)	(0.5)	(3.5)	(176)	(0.19)	(0.44)
Energy	NO_3^-	0.89^{b}	2.34^{b}	7.27^{c}	694^{a}	155^{b}	10.5^{b}	38.3^{a}	1537^{b}	1.12^{b}	1.42^{b}
		(0.02)	(0.12)	(0.26)	(80)	(10)	(0.6)	(3.5)	(180)	(0.06)	(0.40)
	NH_4^+	1.10^{c}	2.95^{c}	8.82^{d}	836^{b}	214^{c}	13.6^{c}	45.3^{a}	1953^{c}	1.00^{b}	1.00^{b}
		(0.03)	(0.21)	(0.09)	(52)	(17)	(0.3)	(8.5)	(255)	(0.15)	(0.46)

The rate of assimilation of macronutrients was always higher for the energy-limited cells. The N source had no effect on the macronutrients assimilation rates, when growth was limited by N; but when energy was limiting, NH_4^+ strongly stimulated the assimilation rates of all macronutrients (included P) (Table 2).

Size of the carbohydrate, lipid and protein pools

Cells cultured in N-limited conditions contained much less proteins, but somewhat more carbohydrates and substantially more lipids than energy-limited cells (Table 1).

Accordingly, when cells were N-limited, the carbohydrate to protein (1.6-fold in NO_3^- and 2.3-fold in NH_4^+), and the lipid to protein (3.5-fold in NO_3^- and 6.7-fold in NH_4^+) ratios were higher than when cells were energy-limited. The carbohydrate to lipid ratio, instead, was 72% (NO_3^-) and 78% (NH_4^+) lower in N-limited than in energy limited cells (Fig. 3).

The N-source made a difference in the biomass composition only when energy was limiting. In these conditions, NO_3^--grown cells contained about 21% less protein than NH_4^+-grown cells. The other main organic pools (carbohydrate and lipid) had similar sizes in the presence of both N-sources, although a high variability was observed in the cell lipid content (Table 1). Consequently, the ratio of carbohydrates to proteins and lipids to proteins was lower in cells grown in NH_4^+ than in NO_3^-; because of the lipid variability, the difference of the lipid to protein ratio was not statistically significant (Fig. 3).

Production rate of biomass, proteins, carbohydrates and lipids

Biomass productivity was significantly lower in N-limited than in energy-limited cells. This effect was mostly due to the different growth rates. The N-source had no impact on biomass productivity in N-limited cells. In energy-limited cells, instead, NO_3^--grown cells had a 36% lower biomass productivity than NH_4^+-grown cells (Table 2). Protein production rate was

Table 2. Rates of C, N, S and P assimilation and rates of production of organic pools and of total biomass, in *Synechococcus* sp. UTEX LB 2380 grown under N or energy limitation The standard deviations are shown in parentheses ($n \geq 3$). Different letters in the superscript identify significantly different means within each group of measurements ($P < 0.05$)

		Macronutrients assimilation rate				Organic pools			
		C	N	S	P	Protein	Carbo- hydrate	Lipid	Biomass production
Limitation	N form	fg·cell^{-1}·d^{-1}	fg·cell^{-1}·d^{-1}	fg·cell^{-1}·d^{-1}	fg·cell^{-1}·d^{-1}	fg·cell^{-1}·d^{-1}	r.u.	r.u.	pg·cell^{-1}·d^{-1}
Nitrogen	NO_3^-	383^a	57.6^a	4.32^a	23.8^a	404^a	1.00^a	1.22^a	0.66 ^{a}
		(50.8)	(8.3)	(0.22)	(2.2)	(73)	(0.04)	(0.05)	(0.07)
	NH_4^+	307^a	41.4^a	4.38^a	19.9^a	405^a	1.03^a	1.25^a	0.49^a
		(56.2)	(8.2)	(0.23)	(1.7)	(85)	(1.13)	(0.19)	(0.09)
Energy	NO_3^-	617^b	138^b	9.36^b	34.1^b	1367^b	1.39^b	1.13^a	2.09^b
		(71.7)	(11.6)	(0.53)	(2.3)	(160)	(0.07)	(0.32)	(0.09)
	NH_4^+	928^c	238^c	15.09^c	50.2^c	2168^c	1.55^b	1.00^a	3.28^c
		(57.5)	(18.9)	(0.30)	(9.5)	(283)	(0.24)	(0.46)	(0.03)

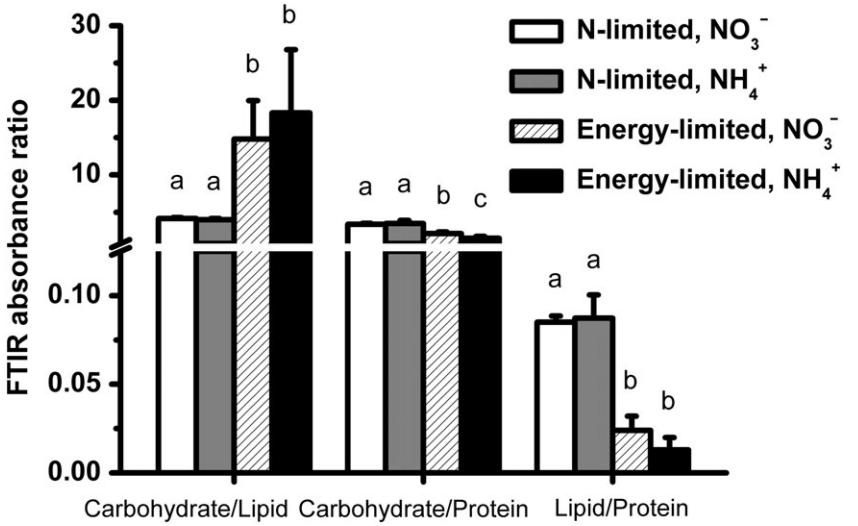

Figure 3. Carbohydrate to lipid, carbohydrate to protein and lipid to protein FTIR absorbance ratios of N-limited and energy-limited *Synechococcus* sp. UTEX LB 2380 cells. Different letters in the superscript denote significantly different means ($P < 0.05$). The error bars indicate the standard deviations ($n \geq 3$).

substantially lower in N-limited cells; this difference was especially evident when NH_4^+ was the N source. The N chemical form only affected protein productivity when energy was limiting. The production rate of carbohydrates was about 30% lower in N-limited than in energy-limited cells, both in NO_3^- and in NH_4^+. Lipid productivity was not affected by either the type of limitation or the chemical form in which N was supplied (Table 2).

Photosynthetic oxygen evolution

O_2 evolution was lower in the N-limited cells than in the energy-limited cells. Under energy limitation, the O_2 evolution rate was higher in the presence of NH_4^+ than of NO_3^-, while the opposite was true when N was limiting: in this case, the photosynthetic rate was higher in the presence of NO_3^- (Fig. 4).

DISCUSSION

Cell stoichiometry

C and N assimilation are usually tightly coupled (Kaffes *et al.* 2010); this constrains cell stoichiometry within fairly strict limits (Giordano 2013); however, changes in resource use efficiency (e.g. Beardall & Giordano 2002; Raven *et al.* 2012) or severe nutrient limitation or excess can lead to diversions from the optimal stoichiometry (Giordano 2013). Furthermore, energy limitation, because of the different costs for the assimilation of the various nutrients, can influence the relative abundance of elements (Kana & Glibert 1987). Unfortunately, although numerous papers report elemental cell stoichiometries, the information on energy availability and on growth limitation is not always available. Our data show that N growth concentration had an obvious repercussion on the C/N and N/P ratio of *Synechococcus* cells, but cell stoichiometry was also directly affected by energy availability. This is demonstrated by the fact that only when energy was limiting, a selective use of the cheaper N source was enacted and that this led to a different C/N ratio and N quota between NH_4^+ and NO_3^--grown cells. Instead, in N-limited cells, which exerted no obvious preference for either N source (if anything, they appear to have taken up slightly more N in the presence of NO_3^-), elemental stoichiometry was unaffected by the N source.

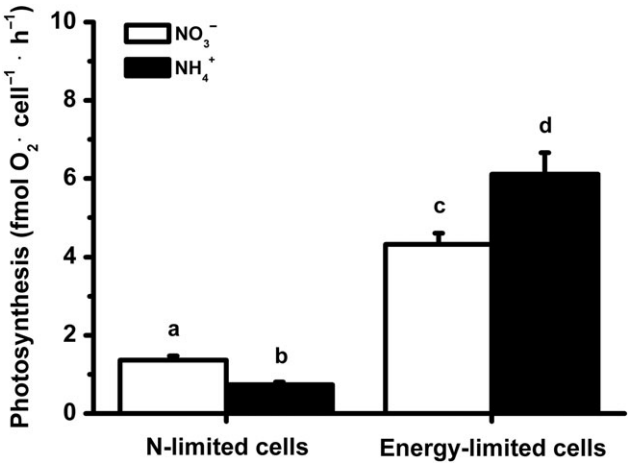

Figure 4. Photosynthesis at growth irradiance (100 μmol photons·m^{-2}·s^{-1}) of N-limited and energy-limited *Synechococcus* sp. UTEX LB 2380 cells. Different letters in the superscript denote significantly different means ($P < 0.05$). The error bars indicate the standard deviations ($n \geq 3$).

A rough calculation, using the information in Tables 3, 4 and 5, allows estimating that, in our experiments, when energy was limiting growth, NH_4^+-grown cells used 58% less energy than NO_3^--grown cells, although they contained 38% more N. Thus, the proportion of energy spent for N assimilation was 24% of the total when NO_3^- was the N source, and only 10% when N was supplied as NH_4^+ (Table 5). It is noteworthy that the 58% difference between the energy for the assimilation of N by NH_4^+ and NO_3^--grown cells matches (in the reverse direction) the difference in the energy invested for the assimilation of the other macronutrients (Tables 4 and 5). As a matter of fact, most of this energy goes into additional C and S assimilation, because the energy used for P was not significantly affected by the N-source. The energy saved by using NH_4^+ rather than NO_3^- is mostly constituted by reducing power (Table 3); it is therefore not surprising that it is utilized for the assimilation of C and S and not for that of P: the latter, in fact, does not impinge appreciably on reducing power, whereas C, N and S assimilations constitute the main sinks for the cell reducing power. This may have played a role in the relative homeostasis of P cell quota in our cells. It is however also known that algae and (especially) cyanobacteria perform P luxury uptake (Kornberg *et al.* 1999; Bertilsson *et al.* 2003; Schwarz & Forchhammer 2005; Zhu *et al.* 2015), which often uncouples P quotas from strict stoichiometric relationships with the other nutrients and from growth (Flynn *et al.* 2010; Giordano *et al.* 2015).

The N/S ratio showed some interesting trends: at limiting N, this ratio was much lower in the presence of NH_4^+ than NO_3^-, for reasons that our data do not allow to fully elucidate. At limiting energy, however, the N/S ratio of NH_4^+-grown cells was much higher than under N-limitation, because of the fact that the N quota increased more than the S quota. It is interesting that the S cell quota increased under energy limitation, especially when NH_4^+ was the N source, although less than the N cell quota. Sulphate assimilation, per mole of substrate, is nearly as expensive as the assimilation of NO_3^- (Table 3), even if elemental stoichiometry makes its absolute cost per cell fairly minor relative to the total cost of macronutrient assimilation (Table 5). Sulphur is an essential component of cells, involved in pivotal functional roles (Giordano & Raven 2014; Giordano & Prioretti 2016; Prioretti & Giordano 2016); therefore, even when energy is limiting, it may not be possible to curb SO_4^{2-} assimilation, which may in fact be required in higher amounts because of the concomitant increase in protein. When S increases, N increases by an extent that maintains the approximate molar ratio between the increment of N and that of S always around 40. If the N:S molar ratio of protein is 62.5, as proposed by Geider & La Roche (2002), a substantial excess of N is available for the biosynthesis of nucleic acids and other non-proteinaceous N-containing compounds. If instead the protein N:S stoichiometry is 39, as reported by Fraga (2001)

Table 3. Minimum energy cost for C, N, S and P assimilation. The energy of two electrons from the oxidation of one NAD(P)H or two ferredoxin was assumed equivalent to two ATP (Raven *et al.* 2000; Lavoie *et al.* 2016). It was also assumed that one ATP is required to pump 1 H^+ (Sanders & Bethke 2000; Andrews *et al.* 2005). The processes are not shown as complete chemical reaction, but solely intend to indicate the electrons and ATP involved

Nutrients	Process of uptake and assimilation	Number of ATP equivalents	References
C	CO_2 uptake	1	Raven *et al.* (2014)
	$CO_2 + 4e + 3ATP \rightarrow 1/3$ triose-Pi + 3ADP	7	Turpin (1991) Raven *et al.* (2014)
	Total cost	8	
N	NO_3^- uptake	2	Andrews *et al.* (2005)
S	$1NO_3^-/2H^+$ symport		
	NH_4^+ uptake	1	Ortiz-Ramirez *et al.* (2011)
	$1NH_4^+/1H^+$ antiport		Turpin (1991)
	$NO_3^- + 8\ e \rightarrow NH_4^+$	8	Guerrero *et al.* (1981)
	NH_4^+ + 2-oxoglutarate + 2e + 1ATP → Glu + 1ADP + 1Pi	3	Cullimore and Sims (1981); Vega et al. (1987) Turpin (1991)
	Total cost	13 (NO_3^-) 4 (NH_4^+)	
	SO_4^{2-} uptake	1	Ritchie (1996)
	$1ATP/1SO_4^{2-}$		
	$SO_4^{2-} + 8e + ATP \rightarrow S^{-2} + ADP$	9	Bick and Leustek (1998); Crawford *et al.* (2000);
	S^{-2} + *O*-acetylserine + ATP→ Cysteine + ADP	1	Bick & Leustek (1998); Crawford *et al.* (2000)
	Total cost	11	
P	Pi uptake	2	Gauthier & Turpin (1994);
	Uptake of NH_4^+ by $2H^+$: 1Pi symport		
	R-OH + ATP → R-Pi	1	Falkowski & Raven (2007)
	Total cost	3	

Table 4. Estimates of the minimum amount of energy, as ATP equivalents, used to assimilate C, N, S and P in *Synechococcus* sp. UTEX LB 2380, under N or energy limitation. Ec, energy for C assimilation; EN, energy for N assimilation; E_S, energy for S assimilation; E_P, energy for P assimilation. E_T is the total minimal amount of energy stored ($E_T = E_C + E_N + E_S + Ep$). Means and standard deviation (in parentheses) were calculated from at least three independent cultures. Different letters in the superscript denote significantly different means ($P < 0.05$)

	Minimum costs of energy for macronutrient assimilation (fmol ATP eq. $cell^{-1}$)			
	N-limited cells		Energy-limited cells	
	in NO_3^-	in NH_4^+	in NO_3^-	in NH_4^+
E_C	482^{ab} (*64*)	426^{a} (*78*)	462^{a} (*54*)	558^{b} (*35*)
E_N	101^{a} (*15*)	25^{b} (*5*)	144^{c} (*12*)	61^{d} (*5*)
E_S	2.8^{a} (*0.1*)	3.1^{a} (*0.2*)	3.6^{b} (*0.2*)	4.7^{c} (*0.1*)
E_P	4.3^{a} (*0.4*)	4.0^{a} (*0.3*)	3.7^{a} (*0.3*)	4.4^{a} (*0.8*)
E_T	590^{ab} (*79*)	458^{a} (*83*)	614^{b} (*66*)	628^{b} (*39*)

Table 5. Percentage of cell energy (ATP equivalents) used for C, N, S and P assimilation in *Synechococcus* sp. UTEX LB 2380, under N or energy limitation. Ec, energy for C assimilation; EN, energy for N assimilation; E_S, energy for S assimilation; E_P, energy for P assimilation. E_T is the total minimal amount of energy used for the assimilation of the four macronutrients ($E_T = E_C + E_N + E_S + Ep$). The standard deviations are shown in parentheses ($n \geq 3$). Different letters in the superscript denote significantly different means ($P < 0.05$)

	Percentage of total energy for macronutrient assimilation			
	N-limited cells		Energy-limited cells	
	in NO_3^-	in NH_4^+	in NO_3^-	in NH_4^+
E_C	81.7^{a} (*0.1*)	93.0^{b} (*0.2*)	75.3^{c} (*0.7*)	88.8^{d} (*0.1*)
E_N	17.1^{a} (*0.2*)	5.4^{b} (*0.2*)	23.5^{c} (*0.7*)	9.7^{d} (*0.2*)
E_S	0.48^{a} (*0.05*)	0.71^{ab} (*0.18*)	0.59^{a} (*0.06*)	0.75^{b} (*0.05*)
E_P	0.74^{a} (*0.05*)	0.89^{a} (*0.10*)	0.61^{b} (*0.03*)	0.70^{ab} (*0.17*)
E_T	100	100	100	100

for phytoplankton, then all the additional N and S would be used for protein production. The matter is destined to remain unresolved, until an accurate protein stoichiometry is determined for the same experimental conditions used to generate the data on cell elemental composition.

Energetic considerations

From the stoichiometry determined for our cells (Table 1) and the information provided in Table 3, it can be calculated that the cost to make NO_3^--grown and a NH_4^+-grown cells is very similar. Yet, while the growth rate of N-limited cells was unaffected by the N source, energy-limited cells showed a higher growth rate and biomass production rate when cultured in

NH_4^+. It is worthwhile stressing the fact that even at high N, when the energy supply was increased, the difference in the growth rate of NO_3^- and NH_4^+-grown cells ceased to exist (Fig. 1). The question thus is what allows energy-limited cells to grow faster in NH_4^+ than in NO_3^-. The greater abundance of N in the NH_4^+-grown cells allows an increased protein content. The fact that protein synthesis has priority over other organic pools when energy is limiting was already observed for cyanobacteria (Konopka & Schnur 1980; Coronil *et al.* 1993). From the information in Tables 1 and 3, given an average molar stoichiometry of protein (normalized to N) of 3.82 C : 6.03 H : 1.24 O : 1 N : 0.016 S (recalculated from Geider & La Roche 2002), we can estimate a minimum energy demand for the synthesis of 1 g of protein of about 0.40 and 0.51 ATP equivalents in NH_4^+ and NO_3^-, respectively. For the same amount of available energy, thus approximately 21% more protein can be produced if the N source is NH_4^+ rather than NO_3^-. This calculated value coincides with the 21% difference between the protein content measured in our NH_4^+ and NO_3^--grown energy-limited cells (Table 1). Because about 50% of protein in an algal and cyanobacterial cells are involved in photosynthesis (Kana & Glibert 1987; MacKenzie *et al.* 2005; Raven *et al.* 2013), an increased protein content of cells possibly leads to a stimulation of photosynthesis. The data reported in Fig. 4 indeed show that, at equal PFD, NH_4^+-grown energy-limited cells have a higher photosynthetic rates than their NO_3^--grown counterparts. This may contribute to the functional relationship between protein content and growth rate proposed by Raven (1984b).

Differently from protein, carbohydrate and lipids did not respond to changes in the N source, regardless of what limited growth. It is common knowledge that N limitation, by causing an imbalance in the cell C:N, leads to an increase of carbohydrates and/or lipids (Palmucci *et al.* 2011). The choice of the storage pool can be genotypically determined, but it also depends on external factors (Palmucci *et al.* 2011). Among these factors, energy availability becomes important when energy is limiting, because the cost for the assimilation of C into lipids is approximately 56% higher than that into carbohydrates (Raven 1982; Montechiaro *et al.* 2006; Norici *et al.* 2011). This, in addition of the re-shuffling of pools associate with a more balanced elemental stoichiometry, may constitute an at least partial explanation of why in our experiments (and possibly in other studies), the difference in the lipid pool size between energy sufficient and energy limited cells was much larger than that of carbohydrates (Table 1).

CONCLUSIONS

Our data show that the advantage afforded by the use of NH_4^+ rather than NO_3^- becomes relevant only when energy is limiting. Under these circumstances, the energy saved by the use of the cheaper N source can be invested in other assimilation pathways. Such energy diversion leads to an increased protein cell content, possibly mostly used for photosynthesis, which allows the cells to increase light use efficiency and growth rate.

When N is limiting, the use of the cheaper N source bear no advantage, and cells tend to be more homeostatic.

ACKNOWLEDGMENTS

We thank Antonio Pusceddu for his help for the statistical analyses. This work was funded by grant 27-12-2011 MAE-MOST and by grants GIO2010 and GIO2011 Ricerca Scientifica di Ateneo UNIVPM, all to M.G.

REFERENCES

Agawin N.S.R., Duarte C.M. & Agusti S. (1998) Growth and abundance of *Synechococcus* sp. in a Mediterranean Bay: seasonality and relationship with temperature. *Marine Ecology Progress Series* **170**, 45–53.

Andrews M., Maule H., Raven J. & Mistry A. (2005) Extension growth of impatiens glandulifera at low irradiance: importance of nitrate and potassium accumulation. *Annals of Botany* **95**, 641–648.

Balch W.M., Holligan P.M., Ackleson S.G. & Voss K.J. (1991) Biological and optical properties of mesoscale coccolithophore blooms in the Gulf of Maine. *Limnology and Oceanography* **36**, 629–643.

Beardall J. & Giordano M. (2002) Ecological implications of microalgal and cyanobacterial CO_2 concentrating mechanisms and their regulation. *Functional Plant Biology* **29**, 335–347.

Beardall J., Griffiths H. & Raven J. (1982) Carbon isotope discrimination and the CO_2 accumulating mechanism in *Chlorella emersonii*. *Journal of Experimental Botany* **33**, 729.

Beardall J., Roberts S. & Millhouse J. (1991) Effects of nitrogen limitation on inorganic carbon uptake and specific activity of ribulose-1,5-P2 carboxylase in green microalgae. *Canadian Journal of Botany* **69**, 1146–1150.

Bertilsson S., Berglund O., Karl D.M. & Chisholm S.W. (2003) Elemental composition of marine *Prochlorococcus* and *Synechococcus*: implications for the ecological stoichiometry of the sea. *Limnology and Oceanography* **48**, 1721–1731.

Bick J.A. & Leustek T. (1998) Plant sulfur metabolism – the reduction of sulfate to sulfite. *Current Opinion in Plant Biology* **1**, 240–244.

Collier J.L., Lovindeer R., Xi Y., Radway J.C. & Armstrong R.A. (2012) Differences in growth and physiology of marine *Synechococcus* (cyanobacteria) on nitrate versus ammonium are not determined solely by nitrogen source redox state. *Journal of Phycology* **48**, 106–116.

Coronil T., Lara C. & Guerrero M.G. (1993) Shift in carbon flow and stimulation of amino-acid turnover induced by nitrate and ammonium assimilation in *Anacystis nidulans*. *Planta* **189**, 461–467.

Crawford N., Kahn M., Leustek T. & Long S. (2000) Nitrogen and sulfur. In *Biochemistry and Molecular Biology of Plants* (eds Buchanan B.B., Gruissem W. & Jones R.L.), pp. 786–849. Zanichelli, Berkeley.

Cullimore J.V. & Sims A.P. (1981) Occurrence of two forms of glutamate synthase in *Chlamydomonas reinhardii*. *Phytochemistry* **20**, 597–600.

Domenighini A. & Giordano M. (2009) Fourier transform infrared spectroscopy of microalgae as a novel tool for biodiversity studies, species identification, and the assessment of water quality. *Journal of Phycology* **45**, 522–531.

Falkowski P.G. (1997) Evolution of the nitrogen cycle and its influence on the biological sequestration of CO_2 in the ocean. *Nature* **387**, 272–275.

Falkowski P.G. & Raven J.A. (2007) *Aquatic Photosynthesis*. Princeton University Press, Princeton.

Fanesi A., Raven J.A. & Giordano M. (2014) Growth rate affects the responses of the green alga Tetraselmis suecica to external perturbations. *Plant, Cell and Environment* **37**, 512–519.

Flynn K.J., Raven J.A., Rees T.A.V., Finkel Z., Quigg A. & Beardall J. (2010) Is the growth rate hypothesis applicable to microalgae? . *Journal of Phycology*, **46**, 1–12.

Fraga F. (2001) Phytoplanktonic biomass synthesis: application to deviations from Redfield stoichiometry. *Scientia Marina* **65**, 153–169.

Gauthier D.A. & Turpin D.H. (1994) Inorganic phosphate (Pi) enhancement of dark respiration in the Pi-limited green alga *Selenastrum minutum* (interactions between H^+/Pi cotransport, the plasmalemma H^+-ATPase, and dark respiratory carbon flow). *Plant Physiology* **104**, 629–637.

Geider R. & La Roche J. (2002) Redfield revisited: variability of C:N:P in marine microalgae and its biochemical basis. *European Journal of Phycology* **37**, 1–17.

Giordano M. (1997) Adaptation of *Dunaliella salina* (Volvocales, Chlorophyceae) to growth on NH_4^+ as the sole nitrogen source. *Phycologia* **36**, 345–350.

Giordano M. (2013) Homeostasis: an underestimated focal point of ecology and evolution. *Plant Science* **211**, 92–101.

Giordano M., Beardall J. & Raven J.A. (2005) CO_2 concentrating mechanisms in algae: mechanisms, environmental modulation, and evolution. *Annual Review of Plant Biology* **56**, 99–131.

Giordano M., Kansiz M., Heraud P., Beardall J., Wood B. & McNaughton D. (2001) Fourier transform infrared spectroscopy as a novel tool to investigate changes in intracellular macromolecular pools in the marine microalga *Chaetoceros muellerii* (Bacillariophyceae). *Journal of Phycology* **37**, 271–279.

Giordano M., Palmucci M. & Raven J.A. (2015) Growth rate hypothesis and efficiency of protein synthesis under different sulphate concentrations in two green algae. *Plant, Cell and Environment* **38**, 2313–2317.

Giordano M. & Prioretti L. (2016) Sulphur and algae: metabolism, ecology and evolution. In *The Physiology of Microalgae, Developments in Applied Phycology Series* (eds Borowitzka M.A., Beardall J. & Raven J.A.), pp. 185–209. Springer-Verlag, Berlin Heidelberg.

Giordano M. & Raven J.A. (2014) Nitrogen and sulfur assimilation in plants and algae. *Aquatic Botany* **118**, 45–61.

Guerrero M.G., Vega J.M. & Losada M. (1981) The assimilatory nitrate-reducing system and its regulation. *Annual Review of Plant Physiology* **32**, 169–204.

Hopkinson B.M. & Barbeau K.A. (2008) Interactive influences of iron and light limitation on phytoplankton at subsurface chlorophyll maxima in the eastern North Pacific. *Limnology and Oceanography* **53**, 1303–1318.

Huppe H. & Turpin D. (1994) Integration of carbon and nitrogen metabolism in plant and algal cells. *Annual Review of Plant Biology* **45**, 577–607.

Kaffes A., Thoms S., Trimborn S., Rost B., Langer G., Richter K.U., … Giordano M. (2010) Carbon and nitrogen fluxes in the marine coccolithophore *Emiliania huxleyi* grown under different nitrate concentrations. *Journal of Experimental Marine Biology and Ecology* **393**, 1–8.

Kana T.M. & Glibert P.M. (1987) Effect of irradiances up to 2000 μE m^{-2} s^{-1} on marine *Synechococcus* WH7803-I. Growth, pigmentation, and cell composition. *Deep Sea Research Part A: Oceanographic Research Papers* **34**, 479–495.

Konopka A. & Schnur M. (1980) Effect of light intensity on macromolecular synthesis in cyanobacteria. *Microbial Ecology* **6**, 291–301.

Kornberg A., Rao N.N. & Ault-Riché D. (1999) Inorganic polyphosphate: a molecule of many functions. *Annual Review of Biochemistry* **68**, 89–125.

Kröcher O., Elsener M. & Jacob E. (2009) A model gas study of ammonium formate, methanamide and guanidinium formate as alternative ammonia precursor compounds for the selective catalytic reduction of nitrogen oxides in diesel exhaust gas. *Applied Catalysis B: Environmental* **88**, 66–82.

Kruk C., Martínez A., Nogueira L., Alonso C. & Calliari D. (2015) Morphological traits variability reflects light limitation of phytoplankton production in a highly productive subtropical estuary (Río de la Plata, South America). *Marine Biology* **162**, 331–341.

Kudo I. & Harrison P.J. (1997) Effect of iron nutrition on the marine cyanobacterium *Synechococcus* grown on different N sources and irradiances. *Journal of Phycology* **33**, 232–240.

Lavoie M., Raven J.A. & Levasseur M. (2016) Energy cost and putative benefits of cellular mechanisms modulating buoyancy in aflagellate marine phytoplankton. *Journal of Phycology* **52**, 239–251.

Lowry O.H., Rosebrough N.J., Farr A.L. & Randall R.J. (1951) Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.

MacKenzie T.D.B., Johnson J.M., Cockshutt A.M., Burns R.A. & Campbell D.A. (2005) Large reallocations of carbon, nitrogen, and photosynthetic reductant among phycobilisomes, photosystems, and Rubisco during light acclimation in *Synechococcus elongatus* strain PCC7942 are constrained in cells under low environmental inorganic carbon. *Archives of Microbiology* **183**, 190–202.

Marchetti A., Varela D.E., Lance V.P., Johnson Z., Palmucci M., Giordano M. & Armbrust E.V. (2010) Iron and silicic acid effects on phytoplankton productivity, diversity, and chemical composition in the central equatorial Pacific Ocean. *Limnology and Oceanography* **55**, 11–29.

Mitchell B.G., Brody E.A., Holm-Hansen O., McClain C. & Bishop J. (1991) Light limitation of phytoplankton biomass and macronutrient utilization in the Southern Ocean. *Limnology and Oceanography* **36**, 1662–1677.

Montechiaro F., Hirschmugl C.J., Raven J.A. & Giordano M. (2006) Homeostasis of cell composition during prolonged darkness. *Plant, Cell and Environment* **29**, 2198–2204.

Moore L.R., Post A.F., Rocap G. & Chisholm S.W. (2002) Utilization of different nitrogen sources by the marine cyanobacteria *Prochlorococcus* and *Synechococcus*. *Limnology and Oceanography* **47**, 989–996.

Norici A., Bazzoni A.M., Pugnetti A., Raven J.A. & Giordano M. (2011) Impact of irradiance on the C allocation in the coastal marine diatom *Skeletonema marinoi* Sarno and Zingone. *Plant, Cell and Environment* **34**, 1666–1677.

Ortiz-Ramirez C., Mora S.I., Trejo J. & Pantoja O. (2011) *Pv*AMT1; 1, a highly selective ammonium transporter that functions as H^+/NH_4^+ symporter. *Journal of Biological Chemistry* **286**, 31113–31122.

Palmucci M. & Giordano M. (2012) Is cell composition related to the phylogenesis of microalgae? An investigation using Hierarchical Cluster Analysis of Fourier Transform Infrared spectra of whole cells. *Environmental and Experimental Botany* **75**, 220–224.

Palmucci M., Ratti S. & Giordano M. (2011) Ecological and evolutionary implications of carbon allocation in marine phytoplankton as a function of nitrogen availability: a FTIR spectroscopy approach. *Journal of Phycology* **47**, 313–323.

Partensky F., Hess W. & Vaulot D. (1999) *Prochlorococcus*, a marine photosynthetic prokaryote of global significance. *Microbiology and Molecular Biology Reviews* **63**, 106–127.

Peterson G.L. (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Analytical Biochemistry* **83**, 346–356.

Prioretti L. & Giordano M. (2016) Direct and indirect influence of sulfur availability on phytoplankton evolutionary trajectories. *Journal of Phycology* **in press** .

Ratti S., Giordano M. & Morse D. (2007) CO_2-concentrating mechanisms of the potentially toxic dinoflagellate Protoceratium reticulatum (Dinophyceae, Gonyaulacales). *Journal of Phycology* **43**, 693–701.

Raven J., Kübler J. & Beardall J. (2000) Put out the light, and then put out the light. *Journal of the Marine Biological Association of the UK* **80**, 1–25.

Raven J.A. (1982) The energetics of freshwater algae; energy requirements for biosynthesis and volume regulation. *New Phytologist* **92**, 1–20.

Raven J.A. (1984a) A cost-benefit analysis of photon absorption by photosynthetic unicells. *New Phytologist* **98**, 593–625.

Raven J.A. (1984b) *Energetics and Transport in Aquatic Plants*. Alan R.Liss, New York.

Raven J.A. & Beardall J. (2016) The ins and outs of CO_2. *Journal of Experimental Botany* **67**, 1–13.

Raven J.A., Beardall J. & Giordano M. (2014) Energy costs of carbon dioxide concentrating mechanisms in aquatic organisms. *Photosynthesis Research* **121**, 111–124.

Raven J.A., Beardall J., Larkum A.W.D. & Sanchez-Baracaldo P. (2013) Interactions of photosynthesis with genome size and function. *Philosophical Transactions of the Royal Society, B: Biological Sciences* **368**, 20120264.

Raven J.A. & Giordano M. (2016) Combined Nitrogen. In *The Physiology of Microalgae* (eds Borowitzka A.M., Beardall J. & Raven A.J.), pp. 143–154. Springer International Publishing, Cham.

Raven J.A., Giordano M., Beardall J. & Maberly S.C. (2011) Algal and aquatic plant carbon concentrating mechanisms in relation to environmental change. *Photosynthesis Research* **109**, 281–296.

Raven J.A., Giordano M., Beardall J. & Maberly S.C. (2012) Algal evolution in relation to atmospheric CO_2: carboxylases, carbon-concentrating mechanisms and carbon oxidation cycles. *Philosophical Transactions of the Royal Society, B: Biological Sciences* **367**, 493–507.

Ritchie R. (1996) Sulphate transport in the cyanobacterium *Synechococcus R-2* (*Anacystis nidulans*, *S. leopoliensis*) PCC 7942. *Plant, Cell and Environment* **19**, 1307–1316.

Sanders D. & Bethke P. (2000) Membrane transport. In *Biochemistry and Molecular Biology of Plants* (eds Buchanan B.B., Gruissem W., Vickers K. & Jones R.L.), pp. 110–159. John Wiley & Sons, Berkeley.

Scanlan D.J. (2003) Physiological diversity and niche adaptation in marine *Synechococcus*. *Advances in Microbial Physiology* **47**, 1–64.

Scanlan D.J. (2012) Marine Picocyanobacteria. In *Ecology of Cyanobacteria II: Their Diversity in Space and Time* (ed Whitton B.A.), pp. 503–533. Sprigner Science, New York.

Schwarz R. & Forchhammer K. (2005) Acclimation of unicellular cyanobacteria to macronutrient deficiency: emergence of a complex network of cellular responses. *Microbiology* **151**, 2503–2514.

Sinclair G., Kamykowski D. & Glibert P.M. (2009) Growth, uptake, and assimilation of ammonium, nitrate, and urea, by three strains of *Karenia brevis* grown under low light. *Harmful Algae* **8**, 770–780.

Strom S.L., Macri E.L. & Fredrickson K.A. (2010) Light limitation of summer primary production in the coastal Gulf of Alaska: physiological and environmental causes. *Marine Ecology Progress Series* **402**, 45–57.

Turpin D.H. (1991) Effects of inorganic N availability on algal photosynthesis and carbon metabolism. *Journal of Phycology* **27**, 14–20.

Vega J., Gotor C. & Menacho A. (1987) Enzymology of the assimilation of ammonium by the green alga Chlamydomonas reinhardtii. In *Inorganic Nitrogen Metabolism* (eds Ulrich W., Aparicio P.J., Syrett P.J. & Castillo F.), pp. 132–136. Springer-Verlag, New York.

Vincent W. (2002) Cyanobacterial dominance in the polar regions. In *The Ecology of Cyanobacteria* (eds Whitton B. & Potts M.), pp. 321–340. Kluwer Academica Publisher, New York.

Waite J.N. & Mueter F.J. (2013) Spatial and temporal variability of chlorophyll-*a* concentrations in the coastal Gulf of Alaska, 1998–2011, using cloud-free reconstructions of SeaWiFS and MODIS-Aqua data. *Progress in Oceanography* **116**, 179–192.

Worden A.Z., Nolan J.K. & Palenik B. (2004) Assessing the dynamics and ecology of marine picophytoplankton: the importance of the eukaryotic component. *Limnology and Oceanography* **49**, 168–179.

Zhu S., Wang Y., Xu J., Shang C., Wang Z., Xu J. & Yuan Z. (2015) Luxury uptake of phosphorus changes the accumulation of starch and lipid in *Chlorella* sp. under nitrogen depletion. *Bioresource Technology* **198**, 165–171.

Received 7 September 2016; received in revised form 21 October 2016; accepted for publication 26 October 2016