



## Note

## Nitrogen isotope ratio of cyanobacterial chlorophyll: Chemostat vs. batch culture

Leah E. Tsao<sup>a,b</sup>, Rebecca S. Robinson<sup>c</sup>, Meytal B. Higgins<sup>d</sup>, Ann Pearson<sup>a,\*</sup><sup>a</sup> Department of Earth and Planetary Sciences, Harvard University, Cambridge, MA 02138, USA<sup>b</sup> Department of Biology, Northeastern University, Boston, MA 02115, USA<sup>c</sup> Graduate School of Oceanography, University of Rhode Island, Narragansett, RI 02882, USA<sup>d</sup> Department of Geosciences, Princeton University, Princeton, NJ 08544, USA

## ARTICLE INFO

## Article history:

Received 9 February 2012

Received in revised form 18 May 2012

Accepted 2 June 2012

Available online 9 June 2012

## ABSTRACT

Nitrogen isotope records of chloropigments can be used to reconstruct photic zone processes. However, the  $^{15}\text{N}$  offset between chlorophyll and biomass ( $\varepsilon_{\text{por}} = \delta^{15}\text{N}_{\text{biomass}} - \delta^{15}\text{N}_{\text{chlorophyll}}$ ) of cyanobacteria and eukaryotes grown in laboratory batch cultures differs significantly and the cause of the difference remains unknown. Here, in four experiments with *Synechocystis* sp. PCC 6803, values of  $\varepsilon_{\text{por}}$  were statistically invariant when cultures were maintained at pH 6.5–9.5 under constant growth conditions in a chemostat. The results suggest that the negative values of  $\varepsilon_{\text{por}}$  observed for freshwater cyanobacteria are not artifacts of batch culturing.

© 2012 Elsevier Ltd. All rights reserved.

## 1. Introduction

Nitrogen isotopes in marine sediments are used to study the nutrient cycles of ancient oceans. However, diagenesis may alter the measured values of  $\delta^{15}\text{N}$  of total organic N relative to original biomass (Altabet, 1988; Robinson et al., 2005). Chloropigments unaffected by diagenesis can be used to help interpret sedimentary data (e.g. Chicarelli et al., 1993; Sachs and Repeta, 1999; Kashiyama et al., 2008; Higgins et al., 2012) using the relationship between the  $\delta^{15}\text{N}$  values of chloropigments and the biomass from which they derive. The difference,  $\varepsilon_{\text{por}}$ , averages  $5 \pm 2\%$  for eukaryotic phytoplankton, i.e. chlorophyll is depleted in  $^{15}\text{N}$  by 5‰ (c.f. Sachs et al., 1999). In contrast, the average values of  $\varepsilon_{\text{por}}$  for marine and freshwater cyanobacteria are  $0 \pm 2\%$  and  $-10 \pm 3\%$ , respectively (Beaumont et al., 2000; Higgins et al., 2011), with the sole exception being an outlier sample of marine *Synechococcus* sp. ( $\varepsilon_{\text{por}}$  10‰; Sachs et al., 1999). Of these values, the negative value of  $\varepsilon_{\text{por}}$  for freshwater cyanobacteria is the most unexpected: it indicates a preferential enrichment of  $^{15}\text{N}$  in chlorophyll.

Differences in nutrient sources ( $\text{NH}_4^+$ ,  $\text{NO}_3^-$  and  $\text{N}_2$ ) and light conditions (continuous vs. diel) do not affect the taxonomic patterns of  $\varepsilon_{\text{por}}$  values observed for cyanobacteria (Higgins et al., 2011). However, because all prior studies were conducted in batch culture, it is possible that  $\varepsilon_{\text{por}}$  may be affected by the systematic increase in pH associated with  $\text{CO}_2$  fixation and its impact on  $\text{NH}_4^+ \rightarrow \text{NH}_3$  speciation. The effect of isotopic equilibration on this acid–base pair may be expressed differently between the low

buffer capacity of freshwater media and the high buffering capacity of saltwater, potentially affecting the  $^{15}\text{N}$  content of key biosynthetic intermediates. Alternatively, internal pools of either labile or recalcitrant dissolved organic nitrogen (DON) may accumulate in the batch environment and contribute to isotopic sorting. To determine if unusual values of  $\varepsilon_{\text{por}}$  are an artifactual result of batch conditions, we used a chemostat to maintain cultures of *Synechocystis* sp. PCC 6803, a freshwater taxon, at constant pH and with continuous flow of fresh medium.

## 2. Material and methods

## 2.1. Chemostat set-up

*Synechocystis* sp. PCC 6803 was cultured axenically in a chemostat assembled from 11 polycarbonate bottles (fresh medium, growth chamber and outflow bottle). Sterile lab air ( $0.2\ \mu\text{m}$  Millex-FG50 filter) was supplied continuously via an aquarium pump. The medium was BG-11 + 18 mM  $\text{NO}_3^-$  (Rippka et al., 1979), controlled at a constant inflow/outflow of  $0.6\text{--}0.8\ \text{ml min}^{-1}$  by peristaltic pump (adjusted to maintain constant cell density and growth rate). The culture was illuminated (24 h) with a fluorescent cool white lamp between  $37\text{--}48\ \mu\text{E m}^{-2}\ \text{s}^{-1}$  at a range of  $25.0\text{--}26.7\ ^\circ\text{C}$ . An immersion pH probe (Mettler Toledo 405-DPAS-SC-K8S/150) connected to an Etratron process control pump (DLX-pH-RX/MBB) maintained pH generally within  $\pm 0.1\text{--}0.2$  of the set point by dosing 33 mM HCl as needed (ca.  $20\ \text{ml d}^{-1}$ ;  $0.67\ \text{ml dose}^{-1}$ ). Four experiments (A, B, C, D) were conducted with nominal pH set points of 6.5, 7.5, 8.5 and 9.5, respectively.

\* Corresponding author.

E-mail address: [pearson@eps.harvard.edu](mailto:pearson@eps.harvard.edu) (A. Pearson).

## 2.2. Continuous growth and sampling

Cells were inoculated (1:10) into 800 ml medium and were grown under batch conditions at the selected pH to early log phase (0.2 optical density at 730 nm,  $OD_{730}$ ;  $\mu$  1.5 d<sup>-1</sup>) before initiating pump flow. Flow was adjusted to maintain  $OD_{730}$  0.2 and the culture was grown and flushed until 3–4 replacement volumes of fresh medium had eluted (60 h for **A**, **C** and **D**; 72 h for **B**). Samples were collected at 4 h intervals for the subsequent 12 h for analysis. Culture material from 24, 60 and 84 h was also saved for comparison (collected at 45, 93 and 120 h for **B**). Cells were collected from the outflow bottle – which was maintained at all times on ice (0 °C) in a dark box – and were centrifuged at 4800 rpm, rinsed 2× with 10 ml Nanopure® H<sub>2</sub>O, pelleted and stored frozen (–20 °C).

## 2.3. Chlorophyll extraction and separation

Pellets were extracted in 10 ml dichloromethane (DCM)/MeOH (2:1) by vortexing and sonication according to Higgins et al. (2011). Concentrated extracts were eluted through Na<sub>2</sub>SO<sub>4</sub> and SiO<sub>2</sub> gel pipette columns with 2:1 DCM/MeOH, dried and re-dissolved in 250  $\mu$ l DCM. Extracts were separated using high performance liquid chromatography (HPLC; Agilent 1100 series, diode array UV/Vis) from two injections per sample on a 4.6 × 25 mm ZOBAX SIL column in a gradient from hexane (100%) to MeOH (75%) and EtOAc (25%) at 35.5 min (1 ml/min). Chlorophyll *a* was recovered by way of fraction collection between 8.5 and 10 min.

## 2.4. Chlorophyll oxidation and N isotope analysis

Chlorophyll was converted to NO<sub>3</sub><sup>-</sup> by UV radiation followed by aqueous, alkaline K<sub>2</sub>S<sub>2</sub>O<sub>8</sub> oxidizing reagent (POR; Knapp et al., 2005; Higgins et al., 2009). NO<sub>3</sub><sup>-</sup> was quantified with a chemilumi-

nescent analyzer (Monitor Labs) and 10 nmol N was used to obtain  $\delta^{15}\text{N}$  values via the denitrifier method with *Pseudomonas chlororaphis* and a Thermo Scientific Delta V isotope ratio mass spectrometer (Sigman et al., 2001). NO<sub>3</sub><sup>-</sup> background (0.7 nmol N measured) and a  $\delta^{15}\text{N}$  value of 0 ± 10‰ (assumed) for the oxidation blank were used to correct all  $\delta^{15}\text{N}_{\text{chlorophyll}}$  data with full propagation of error. For  $\delta^{15}\text{N}_{\text{biomass}}$ , aliquots of washed cells were suspended in Nanopure water and transferred to pre-combusted fragments of glass fiber filters inserted in Costech® tin capsules. Capsules were crushed, desiccated and analyzed with a Costech 4010 Elemental Analyzer connected to a Thermo Scientific Delta V mass spectrometer.

## 3. Results and discussion

Growth conditions and isotope results for all experiments are summarized in Table 1. Individual data points for  $\delta^{15}\text{N}_{\text{biomass}}$ ,  $\delta^{15}\text{N}_{\text{chlorophyll}}$  and  $\epsilon_{\text{por}}$  show no significant trends (Fig. 1). The average growth conditions and isotope values for the stabilized experiments (Fig. 1, closed symbols) are: **A**, pH 6.9 ± 0.1,  $\epsilon_{\text{por}}$  -8.6 ± 0.7‰; **B**, pH 7.6 ± 0.0,  $\epsilon_{\text{por}}$  -8.1 ± 1.8‰; **C**, pH 8.6 ± 0.1,  $\epsilon_{\text{por}}$  -9.3 ± 0.5‰; **D**, pH 9.5 ± 0.1,  $\epsilon_{\text{por}}$  -7.8 ± 0.2‰. Most data points from before 64 h or after 72 h (97 h or 105 h for **B**; Fig. 1, open symbols) were also not outside these ranges and the average propagated error of measurement for individual  $\epsilon_{\text{por}}$  values ( $1\sigma = \pm 0.8\%$ ) is larger than the standard deviation within most experiments and the differences between experiments (Table 1).

Critically, to change the value of  $\epsilon_{\text{por}}$ , any causative process must affect incorporation of <sup>15</sup>N into pigment N differently from whole protein (biomass) N. This requirement is difficult to satisfy, because in cyanobacteria, chloropigments are produced from the central amino acid glutamate, suggesting pigment and protein  $\delta^{15}\text{N}$  values should covary.

In batch growth, it is conceivable that  $\epsilon_{\text{por}}$  could be affected as a function of pH due to the equilibrium fractionation factor between NH<sub>4</sub><sup>+</sup> and NH<sub>3</sub> ( $\alpha_{25^\circ\text{C}}$  1.019; Hermes et al., 1985). NH<sub>3</sub> sits at a lower  $\delta^{15}\text{N}$  value than the system as a whole. Because NH<sub>3</sub> can diffuse through membranes, while NH<sub>4</sub><sup>+</sup> must be transported, the prediction would be that cells would use more diffused NH<sub>3</sub> as pH increases, rather than invest energy in translocating and deprotonating NH<sub>4</sub><sup>+</sup> (the species required for all biosynthetic reactions is NH<sub>3</sub>). If cells preferentially synthesize pigments early in batch culture, drawing on transported (<sup>15</sup>N heavy) N and then recycle those pigments while making <sup>15</sup>N-depleted proteins from diffusive NH<sub>3</sub>,  $\epsilon_{\text{por}}$  might be affected. Such an effect would be most strongly expressed across the pH-pK<sub>a</sub> transition and would be sensitive to the timing of chlorophyll and protein biosynthesis relative to the progression of pH in the culture flask as CO<sub>2</sub> is consumed. It should also be dependent on the relationship between intracellular pH and the pH of the medium and the extent to which reduced N leaks out of cells. However, our chemostat cultures argue against any pH dependent effects on either the absolute values of  $\delta^{15}\text{N}_{\text{biomass}}$  and  $\delta^{15}\text{N}_{\text{chlorophyll}}$  or on values of  $\epsilon_{\text{por}}$  (Fig. 1a and b).

An explanation for <sup>15</sup>N enrichment in chlorophyll of cyanobacteria therefore remains a mystery; however, it appears unlikely that pH provides a first order explanation. An alternative hypothesis might invoke isotopic sorting between cells and extracellular exudates during maturation of batch cultures, but again, only if such sorting occurred in tandem with differential timing of pigment and bulk protein synthesis. Experimenting under chemostat conditions also can address this possibility. Unlike the cultures of Higgins et al. (2011), which grew for an average of ca. 10 days in a single batch of medium before sampling, the residence time ( $\tau$ ) of medium in the chemostat cultures was 17–22 h. Our samples were taken at ca. 4 $\tau$ , or nearly steady-state conditions; the samples

**Table 1**

Raw data. Errors for measurements are reported as  $\pm 1\sigma$  of replicate measurements with propagation of error from blank corrections, unless the value is less than the estimated  $\pm 0.5\%$  absolute uncertainty of the instrument performance, in which case  $\pm 0.5\%$  is substituted. Values in italics are for samples pre- or post-equilibration of the culture in the chemostat; values in normal text are for optimized, stable samples collected after 3–4 $\tau$  equilibration time.

pH	(h)	$\delta^{15}\text{N}_{\text{biomass}}$ (‰)	$\delta^{15}\text{N}_{\text{chl}a}$ (‰)	$\epsilon_{\text{por}}^a$ (‰)
6.70	24	5.4 ± 0.3	13.7 ± 0.5	-8.3 ± 0.6
6.83	60	5.4 ± 0.3	14.0 ± 0.5	-8.6 ± 0.6
6.81	64		13.2 ± 1.0	-8.0 ± 1.0
6.85	68		13.6 ± 0.5	-8.4 ± 0.6
6.91	72		14.6 ± 0.5	-9.4 ± 0.6
6.76	84	5.0 ± 0.4	13.9 ± 0.8	-8.9 ± 0.9
7.53	45	6.3 ± v	17.3 ± 0.5	-11.0 ± 0.6
7.22	93	6.5 ± 0.0	14.9 ± 0.7	-8.4 ± 0.7
7.58	97		15.7 ± 0.5	-9.3 ± 0.5
7.57	101		12.4 ± 0.5	-5.9 ± 0.5
7.56	105		15.4 ± 0.6	-9.0 ± 0.6
7.61	117	6.4 ± 0.2	14.4 ± 0.9	-8.1 ± 0.9
8.31	24	6.6 ± 0.6	16.1 ± 0.5	-9.5 ± 0.8
8.68	60	6.0 ± 0.4	13.2 ± 0.5	-7.2 ± 0.6
8.66	64		15.6 ± 0.5	-9.6 ± 0.6
8.52	68		14.8 ± 0.7	-8.8 ± 0.8
8.47	72		15.7 ± 0.5	-9.7 ± 0.6
8.63	84	6.1 ± 0.3	15.4 ± 0.8	-9.3 ± 0.8
9.52	24	5.9 ± 0.3	12.8 ± 1.4	-6.9 ± 1.5
9.25	60	6.2 ± 0.9	13.3 ± 0.5	-7.1 ± 1.0
9.43	64		14.1 ± 0.5	-7.8 ± 1.0
9.50	68		14.2 ± 0.6	-8.0 ± 1.1
9.56	72		13.9 ± 0.6	-7.7 ± 1.0
9.51	84	6.3 ± 0.6	11.9 ± 0.5	-5.6 ± 0.8

<sup>a</sup>  $\delta^{15}\text{N}_{\text{biomass}}$  not available, calculated relative to average of preceding and lagging values.

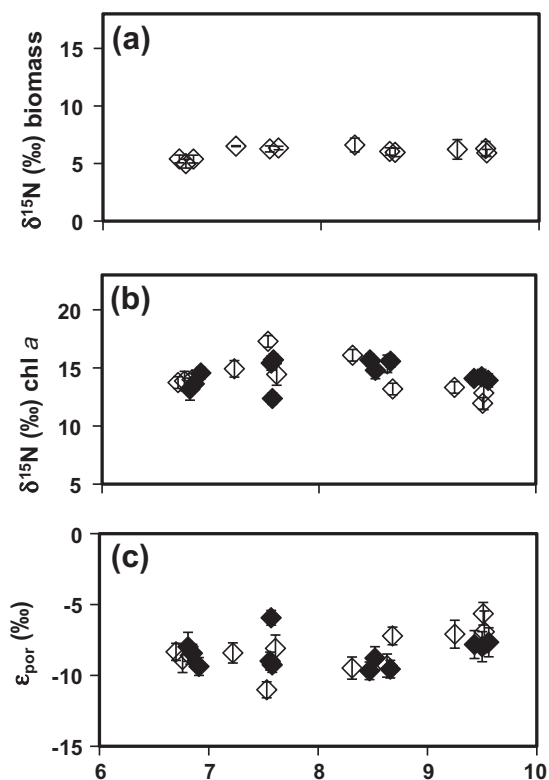


Fig. 1. Values of  $\delta^{15}\text{N}$  for total biomass (a), chlorophyll *a* (b) and  $\epsilon_{\text{por}}$  values (c). Open symbols correspond to pre- and post-equilibration samples. Closed symbols correspond to optimized, stable samples.

should contain less exudate than a batch culture. *Synechocystis* sp. 6803 in mid-log phase has an  $\text{OD}_{730}$  of ca. 0.6, whereas the samples collected here ( $\text{OD}_{730}$  0.2) were in early stage of growth with unlimited nutrient source and without an accumulation of waste products. By itself, this condition would not necessarily rule out the question of isotopic sorting. However, when compared with the  $\epsilon_{\text{por}}$  data reported by Higgins et al. (2011), the results from the two approaches are similar:  $\epsilon_{\text{por}}$  values averaged ca.  $-12\text{‰}$  in the batch work and ca.  $-8.5\text{‰}$  here. If the difference in  $\epsilon_{\text{por}}$  values between freshwater cyanobacteria in batch cultures ( $\epsilon_{\text{por}}$  ca.  $-12\text{‰}$ ) and eukaryotes ( $\epsilon_{\text{por}}$  ca.  $5\text{‰}$ ) was due to accumulation of exudate, substituting a chemostat for batch culturing should have erased most of this  $17\text{‰}$  gap. Instead, the switch to chemostat culturing erased only ca. 20% of the effect. The difference between the two approaches does appear to be significant, however, as the batch culture  $\epsilon_{\text{por}}$  value is significantly more negative than the values here.

Differences in growth rate between batch and chemostat cultures also may affect the magnitude of  $\epsilon_{\text{por}}$ . If so, understanding the interplay between growth rate and the relative timing of chlorophyll synthesis might help explain why  $\epsilon_{\text{por}}$  values for freshwater cyanobacteria are negative. If growth rate were important, culture data would be difficult to extend to environmental interpretations, although the one reported measurement of  $\epsilon_{\text{por}}$  for a freshwater system indicates that it has a negative value in the environment as well (Katase and Wada, 1990). The doubling time in our study was 17–22 h in the chemostats, while the starter cultures and most of the batches reported by Higgins et al. (2011) had a doubling time from 24–48 h; both are within the range of the natural doubling time of *Synechocystis* sp. 6803. Across these rates, it appears that  $\epsilon_{\text{por}}$  is minimally affected, although both slower and faster rates should be explored in the future. In batch cultures, a decreased growth rate in late log phase and stationary phase might

allow increased utilization of the lighter N isotope for biomass as a whole. This could produce more isotopically divergent samples if biomass and pigments are synthesized from different pools of N, or at different relative rates throughout the course of a batch culture, or are affected by changing the proportion of fluxes of key intermediate(s) around a biosynthetic branching point (Hayes, 2001). Chemostats should diminish the effect of temporal sorting and bring the  $\delta^{15}\text{N}$  values of chlorophyll and biomass closer together, as observed. However, the observations reported here suggest that the changes induced by chemostat approaches relative to batch culturing are minor. Clearly, more work is needed to decipher why values of  $\epsilon_{\text{por}}$  for freshwater cyanobacteria are so different from other photosynthetic species, but the pattern does not appear to be an artifact of laboratory experimentation.

A final hypothesis to explain negative values of  $\epsilon_{\text{por}}$  could invoke a  $^{15}\text{N}$ -fractionating exchange of  $\text{NH}_2$  groups in the glutamate-derived metabolites dedicated to chlorophyll biosynthesis. The N that originates with the glutamate destined to become glutamate-1-semialdehyde and subsequently  $\delta$ -aminolevulinic acid ( $\delta$ -ALA) must somehow become enriched in  $^{15}\text{N}$  relative to the residual glutamate and other amino acids destined for protein synthesis. However, the only N transformation occurs during the transamination reaction, and it is believed that this reaction conserves  $\text{NH}_3$ . The N that is transferred from the pyridoxamine cofactor of glutamate-1-semialdehyde aminotransferase to form the  $\delta$ - $\text{NH}_2$  group of  $\delta$ -ALA is replaced (reversion of pyridoxal to pyridoxamine) using the  $\alpha$ - $\text{NH}_2$  from the proximal glutamate-1-semialdehyde (Mayer et al., 1993; Ge et al. (2010)). This is an intermolecular transfer that is thought to inhibit exchange of  $\text{NH}_2$  groups with exogenous  $\text{NH}_3$ . Therefore, the only opportunity for fractionation appears to lie within a biosynthetic branch point affecting the original pool of glutamate itself. Future work is needed to examine the  $\delta^{15}\text{N}$  values of subcellular pools of glutamate and glutamate-1-semialdehyde, both in cyanobacteria and in other photosynthetic species.

## Acknowledgments

We thank W. Leavitt for chemostat advice, and S. Carter and R. Bovee for laboratory assistance. The work was sponsored by the NASA Astrobiology Institute-MIT consortium, NSF-OCE and Harvard University. The anonymous reviewer is thanked for constructive comments.

Associate Editor—K. Grice

## References

- Altabet, M.A., 1988. Variations in nitrogen isotopic composition between sinking and suspended particles – implications for nitrogen cycling and particle transformation in the open ocean. *Deep Sea Research Part A – Oceanographic Research Papers* 35, 535–554.
- Beaumont, V.I., Jahnke, L.L., Des Marais, D.J., 2000. Nitrogen isotopic fractionation in the synthesis of photosynthetic pigments in *Rhodospirillum rubrum* and *Anabaena cylindrica*. *Organic Geochemistry* 31, 1075–1085.
- Chicarelli, M.L., Hayes, J.M., Popp, B.N., Eckardt, C.B., Maxwell, J.R., 1993. Carbon and nitrogen isotopic compositions of alkyl porphyrins from the Triassic Serpiano oil shale. *Geochimica et Cosmochimica Acta* 57, 1307–1311.
- Ge, H., Lu, X., Fan, J., Gao, Y., Teng, M., Niu, L., 2010. Crystal structure of glutamate-1-semialdehyde aminotransferase from *Bacillus subtilis* with bound pyridoxamine-5'-phosphate. *Biochemical and Biophysical Research Communications* 402, 356–360.
- Hayes, J.M., 2001. Fractionation of carbon and hydrogen isotopes in biosynthetic processes. *Reviews in Mineralogy and Geochemistry* 43, 225–277.
- Hermes, J.D., Weiss, P.M., Cleland, W.W., 1985. Use of  $^{15}\text{N}$  and deuterium isotope effects to determine the chemical mechanism of phenylalanine ammonia lyase. *Biochemistry* 24, 2959–2967.
- Higgins, M.B., Robinson, R.S., Casciotti, K.L., McIlvin, M.R., Pearson, A., 2009. A method for determining the nitrogen isotopic composition of porphyrins. *Analytical Chemistry* 81, 184–192.

- Higgins, M.B., Wolfe-Simon, F., Robinson, R.S., Qin, Y.L., Saito, M.A., Pearson, A., 2011. Paleoenvironmental implications of taxonomic variation among  $\delta^{15}\text{N}$  values of chloropigments. *Geochimica et Cosmochimica Acta* 75, 7351–7363.
- Higgins, M.B., Robinson, R.S., Husson, J.M., Carter, S.J., Pearson, A., 2012. Dominant eukaryotic export production during ocean anoxic events reflects the importance of recycled  $\text{NH}_4^+$ . *Proceedings of the National Academy of Sciences USA* 109, 2269–2274.
- Kashiyama, Y., Ogawa, N.O., Shiro, M., Tada, R., Kitazato, H., Ohkouchi, N., 2008. Reconstruction of the biogeochemistry and ecology of photoautotrophs based on the nitrogen and carbon isotopic compositions of vanadyl porphyrins from Miocene siliceous sediments. *Biogeosciences* 5, 797–816.
- Katase, T., Wada, E., 1990. Isolation of chlorophyll-*a* in *Microcystis* spp. for determination of stable isotopes of carbon and nitrogen, and variation in Suwa Lake. *Bunseki Kagaku* 39, 451–456.
- Knapp, A.N., Sigman, D.M., Lipschultz, F., 2005. N isotopic composition of dissolved organic nitrogen and nitrate at the Bermuda Atlantic time-series study site. *Global Biogeochemical Cycles* 19, GB1018.
- Mayer, S.M., Gawlita, E., Avissar, Y.J., Anderson, V.E., Beale, S.I., 1993. Intermolecular nitrogen transfer in the enzymatic conversion of glutamate to  $\delta$ -aminolevulinic acid by extracts of *Chlorella vulgaris*. *Plant Physiology* 101, 1029–1038.
- Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M., Stanier, R.Y., 1979. Generic assignments, strain histories and properties of pure cultures of Cyanobacteria. *Journal of General Microbiology* 111, 1–61.
- Robinson, R.S., Sigman, D.M., DiFiore, P.J., Rohde, M.M., Mashiotto, T.A., Lea, D.W., 2005. Diatom-bound  $15\text{N}/14\text{N}$ : new support for enhanced nutrient consumption in the ice age subantarctic. *Paleoceanography* 20, PA3003.
- Sachs, J.P., Repeta, D.J., 1999. Oligotrophy and nitrogen fixation during eastern Mediterranean sapropel events. *Science* 286, 2485–2488.
- Sachs, J.P., Repeta, D.J., Goericke, R., 1999. Nitrogen and carbon isotopic ratios of chlorophyll from marine phytoplankton. *Geochimica et Cosmochimica Acta* 63, 1431–1441.
- Sigman, D.M., Casciotti, K.L., Andreani, M., Barford, C., Galanter, M., Bohlke, J.K., 2001. A bacterial method for the nitrogen isotopic analysis of nitrate in seawater and freshwater. *Analytical Chemistry* 73, 4145–4153.