Available online at www.sciencedirect.com
ScienceDirect
Geochimica et Cosmochimica Acta 74 (2010) 1030–1040
Geochimica et Cosmochimica Acta
www.elsevier.com/locate/gca

N and O isotope effects during nitrate assimilation by unicellular prokaryotic and eukaryotic plankton cultures

J. Granger [a,*], D.M. Sigman [a], M.M. Rohde [b], M.T. Maldonado [c], P.D. Tortell [c]

[a] Department of Geosciences, Princeton University, Princeton, NJ 08544, USA
[b] School of Geography and the Environment, University of Oxford, Oxford OX1 3QY, United Kingdom
[c] Department of Earth and Ocean Sciences, University of British Columbia, Vancouver, BC, Canada V6T 1Z4

Received 18 May 2009; accepted in revised form 26 October 2009; available online 31 October 2009

Abstract

In order to provide biological systematics from which to interpret nitrogen (N) and oxygen (O) isotope ratios of nitrate ($^{15}N/^{14}N$, $^{18}O/^{16}O$, respectively) in the environment, we previously investigated the isotopic fractionation of nitrate during its assimilation by mono-cultures of eukaryotic algae (Granger et al., 2004). In this study, we extended our analysis to investigate nitrate assimilation by strains of prokaryotic plankton. We measured the N and O isotope effects, $^{15}\varepsilon$ and $^{18}\varepsilon$, during nitrate consumption by cultures of prokaryotic strains and by additional eukaryotic phytoplankton strains (where ε is the ratio of reaction rate constants of the light vs. heavy isotopologues, $^{light}k$ and $^{heavy}k$; $\varepsilon = {}^{light}k/{}^{heavy}k - 1 \times 1000$, expressed in per mil). The observed $^{15}\varepsilon$ ranged from 5‰ to 8‰ among eukaryotes, whereas it did not exceed 5‰ for three cyanobacterial strains, and was as low as 0.4‰ for a heterotrophic α-protoeobacterium. Eukaryotic phytoplankton fractionated the N and O isotopes of nitrate to the same extent (i.e., $^{18}\varepsilon \sim {}^{15}\varepsilon$). The $^{18}\varepsilon$:$^{15}\varepsilon$ among the cyanobacteria was also ~1, whereas the heterotrophic α-proteobacterial strain, which showed the lowest $^{15}\varepsilon$, between 0.4‰ and 1‰, had a distinct $^{18}\varepsilon$:$^{15}\varepsilon$ of ~2, unlike any plankton strain observed previously. Equivalent N vs. O isotope discrimination is thought to occur during internal nitrate reduction by nitrate reductase, such that the cellular efflux of the fractionated nitrate into the medium drives the typically observed $^{18}\varepsilon$:$^{15}\varepsilon$ of ~1. We hypothesize that the higher in the $^{18}\varepsilon$:$^{15}\varepsilon$ of the α-proteobacterium may result from isotope discrimination by nitrate transport, which is evident only at low amplitude of ε. These observations warrant investigating whether heterotrophic bacterial assimilation of nitrate decreases the community isotope effects at the surface ocean.

1. INTRODUCTION

Natural abundance stable isotope ratios provide a powerful tracer to elucidate N cycling in marine environments. In this regard, the N isotope ratios of nitrate have been measured to identify regional nitrate loss by water column denitrification (Cline and Kaplan, 1975; Brandes et al., 1998; Voss et al., 2001), to infer addition of new N from N_2 fixation (Liu et al., 1996; Sutka et al., 2004; Knapp et al., 2005), to provide constraints on regional (Altabet, 1988; Voss et al., 1996) and global ocean (Brandes and Devol, 2002; Deutsch et al., 2004) fixed N budgets, and to determine the community isotope effect for nitrate assimilation in the surface ocean (Wu et al., 1997; Sigman et al., 1999; Altabet, 2001; DiFiore et al., 2006), which assists in paleo-reconstruction of the extent of nitrate consumption in the surface ocean from the sedimentary $\delta^{15}N$ record (François et al., 1997; Robinson et al., 2005).

Until recently, only the N isotopes of nitrate were measured in marine systems due to limitations in methodology (Silva et al., 2000). However, novel methods now enable accurate quantification of both the N and O isotope ratios ($^{15}N/^{14}N$ and $^{18}O/^{16}O$, respectively) of nitrate and nitrite in relatively small freshwater or saline samples (Sigman

* Corresponding author. Fax: +1 609 258 1274.
E-mail address: jgranger@princeton.edu (J. Granger).

0016-7037/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.gca.2009.10.044

et al., 2001; Casciotti et al., 2002; McIlvin and Altabet, 2005; Böhlke et al., 2007). Coupled nitrate N and O isotope measurements are useful because they provide complementary constraints on processes that otherwise erase one another with respect to N isotopes alone. For instance, the $\delta^{15}N$ from nitrate loss by water column denitrification and the addition of isotopically light N by N_2 fixation are processes that work to erase one another with respect to both the concentration of nitrate and its N isotopic composition, when these transformations occur in the same body of water, or when each originates from distinct water masses that are then mixed. Because the O isotopes of nitrate are not sensitive to N_2 fixation, $\delta^{15}N$ values that are lower than would be expected from the proportional $\delta^{15}N$ vs. $\delta^{18}O$ enrichment of nitrate due to denitrification (Granger et al., 2008) may be diagonostic of inputs of new N from nitrogen fixation overlying denitrification zones (Sigman et al., 2005). In addition, the local re-oxidation of nitrite produced by denitrification in oxygen minimum zones may also lower the $\delta^{15}N$ relative to the $\delta^{15}N$ vs. $\delta^{18}O$ expected from denitrification, such that a contribution of nitrite re-oxidation to the nitrate pool can be inferred from the coupled N and O isotope distribution (Sigman et al., 2005). Coupled N and O isotope measurements have also been used to separate nitrification from denitrification in marine sediment (Lehmann et al., 2004, 2005). Similarly, in the surface ocean, coupled N and O isotope measurements may characterize the origin of nitrate, whether supplied directly from depth or regenerated within the surface mixed layer (Wankel et al., 2007).

In order to establish the biological systematics from which to interpret field measurements of coupled nitrate N and O isotopes, we previously investigated nitrate N and O isotope discrimination during nitrate assimilation by mono-cultures of marine unicellular eukaryotic algae (Granger et al., 2004), as well during dissimilatory nitrate reduction by cultures of marine and freshwater denitrifiers (Granger et al., 2008): While the range of N isotope effect amplitudes that was observed for cultures of both assimilators ($^{15}\varepsilon = 5$–$20‰$) and denitrifiers ($^{15}\varepsilon = 3$–$25‰$) was relatively large, O isotope effects were always equivalent to the corresponding N isotope effect. Thus, nitrate N and O isotope ratios appear to co-vary linearly with a constant ratio of ~1 (i.e., $\Delta\delta^{18}O = \Delta\delta^{15}N$) during nitrate assimilation, as well as during respiratory nitrate dissimilation. This biological imprint is reflected in marine environments with observations of a $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ of ~1 associated with nitrate assimilation in the surface ocean (Casciotti et al., 2002), as well as with water column denitrification in oxygen minimum zones (Sigman et al., 2003). The $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ relationship thus serves as an important benchmark to interpret nitrate isotope ratios *in situ*.

Nitrate consumption in the ocean mixed layer is not restricted to eukaryotic phytoplankton but is also carried out by photosynthetic and heterotrophic prokaryotes (Allen et al., 2002 and references therein). Coupled N and O isotope fractionation by prokaryotes has not been studied in culture, such that determination of the N and O isotopic effects associated with nitrate assimilation by prokaryotes is important for interpretation of measurements of nitrate isotope distributions at the surface ocean.

In this study, we present measurements of nitrate N and O isotope fractionation during nitrate assimilation by strains of eukaryotic phytoplankton and prokaryotic unicellular plankton. Our results suggest that prokaryotes cover a narrower range of amplitude of $^{15}\varepsilon$ than observed among eukaryotes, which has implications for interpretation of the community isotope effect during nitrate assimilation in the surface ocean. Yet, like eukaryotes, prokaryotes generally conform to a nominal $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ of 1 during nitrate assimilation, except for the heterotrophic bacterial strain, which showed distinctively low N and O isotope effect amplitudes, and for which a $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ of ~2 were observed. We interpret these results in the context of the isotope fractionation mechanism during nitrate assimilation, suggesting that the departures in $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ derive from low amplitude isotope fractionation by transport into the cell. Finally, we discuss the implications of the findings for interpretation of measurements of nitrate isotope ratios in the environment.

2. MATERIALS AND METHODS

2.1. Experimental strains

Experimental strains included three marine cyanobacteria, *Synechococcus* sp. WH8108, *Synechococcus* sp. WH7803 (strain DC2), and *Synechococcus* sp. (strain SNC1), two chlorophytes, the freshwater isolates *Chlorella vulgaris* and *Chlorella pyrenoidosa*, two marine diatom species, *Phaedactylum tricornutum* (CCMP630) and *Pseudonitzchia hemii* (isolated at Station P in the North Pacific by A. Marchetti), and a heterotrophic α-proteobacterium, *Alteromonas macleodii* (strain Jul88, isolated in the Sargasso Sea by C. Suttle and A. Chan).

2.2. Culture conditions

Cells were grown in batch culture. Marine isolates were cultured in filtered surface seawater collected at ocean Station PAPA in the subarctic Pacific, and supplemented with 100 μM nitrate, 10 μM phosphate, and 100 μM silicate. Media were prepared in acid-washed polycarbonate bottles and were sterilized by microwaving (Keller et al., 1988). Sterile seawater was then supplemented with filter-sterilized AQUIL EDTA- (ethylene diamine tetraacetic acid) trace metals and f/2 vitamins (Price et al., 1988/1989). Copper was omitted from cyanobacterial media as even small addition proved toxic and stunted growth. The freshwater chlorophytes, *C. vulgaris* and *C. pyrenoidosa*, were cultured in medium consisting of de-ionized water amended with 5 mM KH_2PO_4, 5 mM K_2HPO_4, 10 mM $Mg_2SO_4{\cdot}7H_2O$, 0.5 mM $CaCl_2{\cdot}2H_2O$, 11 μM H_2BO_3, and supplemented with 100 μM nitrate, AQUIL EDTA-trace metals and f/2 vitamins. *A. macleodii* was cultured in algal seawater medium supplemented with 0.5 g L^{-1} glucose, 0.5 g L^{-1} sodium acetate, and 100 μM nitrate as its sole nitrogen source.

The diatoms and chlorophytes were grown under continuous light at 20 °C. The cyanobacteria cultures failed

to grow under continuous light and were thus grown on the bench-top and subject to daily changes in light intensity and temperature in the lab. The heterotrophic bacterium *A. macleodii* was also grown on the bench-top.

Growth of algal strains was monitored by fluorescence measurements on a Turner AU-10 fluorometer, whereas growth of *A. macleodii* was determined from turbidity measurements at 660 nm on a CARY IE UV–Vis (Varian) spectrophotometer.

2.3. Sample collection and isotope analyses

Throughout exponential growth of the cultures, 20 mL sub-samples were collected for nitrate N and O isotope analyses. These were filtered through a pre-rinsed 0.2 μm pore-size polyethylene-sulfone membrane filter to remove cells, and filtrates were stored in acid-washed polypropylene bottles that were frozen until nitrate concentration analysis and subsequent nitrate N and O isotope analyses. Nitrate concentration was measured by conversion to NO (nitric oxide) in hot vanadium (III) solution followed by chemiluminescence detection (Braman and Hendrix, 1989) on an Antek 1750 nitrate/nitrite analyzer.

The $^{15}N/^{14}N$ and $^{18}O/^{16}O$ of nitrate were determined following the denitrifier method (Sigman et al., 2001; Casciotti et al., 2002). Briefly, nitrate in individual samples was converted quantitatively to N_2O gas by denitrifying bacteria that lack a terminal N_2O reductase. Automated extraction of the N_2O analyte from sample vials was achieved on an adapted Finnigan GasBenchII (Casciotti et al., 2002), in line with an isotope ratio mass spectrometer (Finnigan DELTA^{plus}) operated in continuous flow mode to measure the $m/z = 45/44$ and 46/44 ratios of N_2O. Isotope ratios are reported using the delta (δ) notation in units of per mil (‰):

$$\delta^{15}N_{sample} = [(^{15}N/^{14}N)_{sample}/(^{15}N/^{14}N)_{reference} - 1] \times 1000 \quad (1a)$$

$$\delta^{18}O_{sample} = [(^{18}O/^{16}O)_{sample}/(^{18}O/^{16}O)_{reference} - 1] \times 1000 \quad (1b)$$

The $^{15}N/^{14}N$ reference is N_2 in air, and the $^{18}O/^{16}O$ is Vienna standard mean ocean water (VSMOW). Individual analyses were referenced to injections of N_2O from a pure gas cylinder and then standardized through comparison to the international potassium nitrate reference materials IAEA-N3 with an assigned $\delta^{15}N$ of +4.7‰ vs. atmospheric N_2 (Gonfiantini et al., 1995) and most recently reported $\delta^{18}O$ of +25.6‰ vs. SMOW (Böhlke et al., 2003) and nitrate isotopic standard USGS-34 ($\delta^{15}N = -1.8$‰, $\delta^{18}O = -27.9$‰; Böhlke et al., 2003). The size of the culture blank was determined by running a prepared vial to which no sample was added. The N and O isotopic measurements of roughly 50% of the samples were replicated in separate batch analyses. Replicate isotopic measurements of individual samples were generally 0.5‰ for $\delta^{15}N$ and 0.8‰ for $\delta^{18}O$: We have noted that our measurement error in replicate analyses of some culture samples is noticeably higher than that generally obtained for environmental samples (namely, 0.2‰ for N and 0.5‰ for O), yet we remain uncertain as to the cause of this variability. As the current samples contained no detectable nitrite, whose isotopic composition is not stable during storage (Casciotti et al., 2007), we suspect that active enzymes or reactive organometallic complexes may remain in sample filtrates, which could alter the isotopic composition of nitrate during storage, or interfere with the bacterial steps of N_2O production during sample conversion.

To derive estimates of the N and O isotope effects imparted on nitrate ($^{15}\varepsilon$ and $^{18}\varepsilon$, respectively), nitrate $\delta^{15}N$ and $\delta^{18}O$ measurements were fit to the following Rayleigh linearization, where the slope approximates ε (Mariotti et al., 1981):

$$\delta^{15}N = \delta^{15}N_{initial} + {}^{15}\varepsilon\{\ln([NO_3^-]/[NO_3^-]_{initial})\} \quad (2a)$$

$$\delta^{18}O = \delta^{18}O_{initial} + {}^{18}\varepsilon\{\ln([NO_3^-]/[NO_3^-]_{initial})\} \quad (2b)$$

Given the relatively small amplitude of isotope effects observed in this study, this linear approximation gives the same results within experimental error as the exact Rayleigh expression (Mariotti et al., 1981). Regression slopes and associated statistical errors were determined by fitting the data with major axis regression analyses, in which normal deviates of both x and y coordinates are minimized in order to approximate an optimal linear fit.

3. RESULTS

The exponential growth rates observed among the eukaryotic phytoplankton strains were roughly similar, from 1.0 to 1.5 d^{-1} (data not shown). Growth rates among the cyanobacteria were generally slower, from 0.5 to 0.9 d^{-1}. The fastest growth rates were observed for the heterotrophic prokaryote *A. macleodii*, ranging between 6 and 7 d^{-1}. Nitrate, which constituted the sole nutritional N source, was consumed in proportion to growth of the cultures (Fig. 1a). The $\delta^{15}N$ and $\delta^{18}O$ of nitrate increased concomitantly as nitrate was depleted (Fig. 1b).

The N isotope effects observed among the experimental strains spanned values ranging between 0.4‰ and 8‰ (Table 1, Fig. 2). Eukaryotes showed isotope effects between 5‰ and 8‰, and cyanobacteria between 2‰ and 5‰. The heterotrophic prokaryote *A. macleodii* displayed the lowest $^{15}\varepsilon$, which ranged between 0.4‰ and 1‰.

Among the eukaryotic strains examined, O isotope effects were generally close to corresponding N isotope effects, with a $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ ranging between 0.9 and 1.2, and showed no apparent systematic differences among strains (Table 1; Fig. 3a). Similarly, a $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ of ~1 also characterized the cyanobacterial strains, except perhaps for the single culture of *Syn.* sp. SNC1, which showed an arguably distinct $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ of ~1.3 (Table 1; Fig. 3a and b). Strain SNC1 grew poorly under our culture conditions such that we were unable to provide isotope analyses from replicate cultures.

The α-proteobacterium *A. macleodii*, however, showed $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ trajectories that were unmistakably greater than 1, clustering around a distinct value of ~2 for all experimental cultures (Table 1; Fig. 3b).

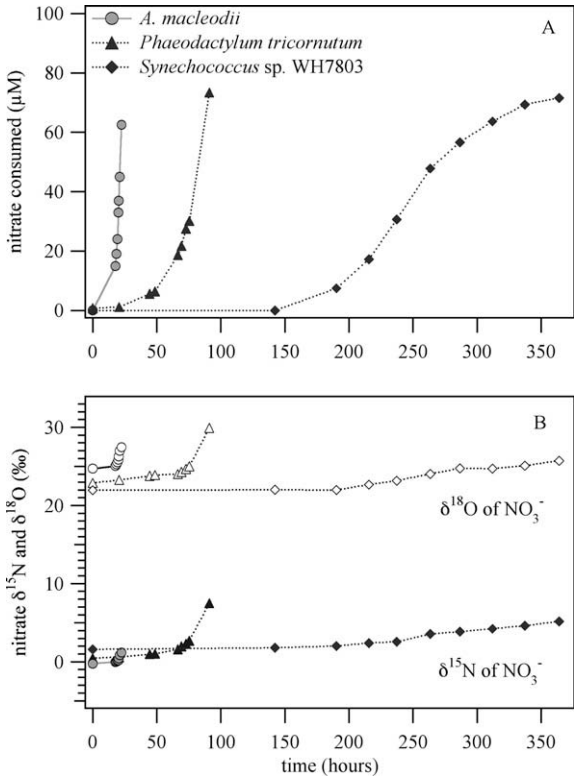

Fig. 1. (A) Nitrate consumed as a function of time for three representative plankton cultures and (B) concomitant change in the N and O isotope composition of nitrate.

4. DISCUSSION

This study uncovers important insights on nitrate isotope fractionation by prokaryotic vs. eukaryotic plankton. First, while the N (and O) isotope effects appeared variable among all experimental strains, the results suggest that prokaryotes may express a lower or more restricted range of N and O isotope effects than eukaryotic strains. We investigate whether this pattern is further confirmed in the extant literature. The physiological mechanisms underlying variations in the isotope effect amplitude are also explored. Secondly, the current results provide important confirmation that the previously documented $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ of ~1 for eukaryotes extends to prokaryotic algae. This homology in isotope fractionation is examined in the context of the isotope fractionation mechanism, and with respect to the phylogeny and the documented structural heterogeneity of nitrate reductase enzymes of prokaryotes and eukaryotes. Thirdly, the results reveal a divergence of the heterotrophic prokaryote from a nominal $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ of ~1. The potential physiological mechanisms that contribute to this distinction are explored. And finally, we consider the contribution of autotrophic and heterotrophic prokaryotes to the community isotope effect of nitrate assimilation in the surface ocean, as well as the implications of the current results for interpretation of nitrate N and O isotope distributions in the environment.

4.1. Physiological constraints on the N isotope effect amplitude

The $^{15}\varepsilon$ values observed here for cyanobacteria ($\leqslant$5‰) are in keeping with previously reported values of 5‰ and 3‰ for a marine and a freshwater *Synechococcus*, respectively (Shearer et al., 1991; Needoba et al., 2003). The $^{15}\varepsilon$ values observed among the eukaryotes in this study (i.e., 5–8‰), namely two diatoms and two chlorophytes, also clearly fall within the broad range reported previously for eukaryotic phytoplankton of 1–20‰, though commonly below 10‰ (Wada and Hattori, 1978; Montoya and McCarthy, 1995; Pennock et al., 1996; Waser et al., 1998; Needoba et al., 2003; Granger et al., 2004; Needoba and Harrison, 2004). Comparatively, cyanobacteria appear to be restricted to a narrower and lower range in $^{15}\varepsilon$ ($\leqslant$5‰) than eukaryotes as a group, though a more comprehensive sampling effort of cyanobacterial strains could admittedly prove otherwise. Notwithstanding, the N isotope effects observed for *A. macleodii* are comparatively very low, and could be characteristic of isotope effects among heterotrophic prokaryotes in general.

The differences in $^{15}\varepsilon$ among plankton strains ostensibly reflect differences in the regulation of nitrate metabolism. During nitrate assimilation, transport into the cells imparts minor isotope discrimination on nitrate, whereas subsequent reduction to nitrite by nitrate reductase imparts significant isotopic discrimination on internal nitrate (Shearer et al., 1991; Granger et al., 2004; Needoba et al., 2004), with an enzymatic N isotope effect of 15–25‰ (Ledgard et al., 1985; Schmidt and Medina, 1991; Needoba et al., 2004). The internal isotopic enrichment propagates to the external nitrate pool by cellular nitrate efflux, such that the magnitude of the N (and O) isotope effects measured in external nitrate ($^{15}\varepsilon$ and $^{18}\varepsilon$) reflect the fraction of the enzymatic isotope discrimination that is transmitted to the external pool as a function of the ratio of cellular nitrate efflux to gross nitrate uptake (Fig. 4; Shearer et al., 1991; Granger et al., 2004; Needoba et al., 2004). In that respect, *A. macleodii*, which shows little N isotope enrichment of external nitrate, is effectively subject to limited cellular nitrate efflux, which signifies that the rate of internal nitrate reduction during the growth of *A. macleodii* is roughly similar to its rate of nitrate uptake.

While we can identify the mechanisms that give rise to different amplitudes of N isotope effects, the physiological or ecological impetus for differential regulation of nitrate metabolism among cultures remains unclear. At the outset, there is no established role for nitrate efflux in microbial physiology (Needoba et al., 2004), whether efflux consists of passive diffusion through non-specific ion channels, or whether it is a strictly regulated reverse flow that serves in electrochemical balancing or that is used to power the uphill movement of other ions through antiporters at the cell membrane. On the second hand, there do not appear to be robust distinctions among clades with respect to the regulation of nitrate metabolisms as captured by the isotope effect amplitude (Wada and Hattori, 1978; Montoya and McCarthy, 1995; Needoba and Harrison, 2004). Similarly, isotope effects can be widely different within strains, in spite of

Table 1
$^{15}\varepsilon$ and $^{18}\varepsilon$ (‰) observed during nitrate assimilation among experimental cultures, and the corresponding $\Delta\delta^{18}O{:}\Delta\delta^{15}N$. Isotope effects were determined from the slope of linear regressions of $\delta^{15}N$ and $\delta^{18}O$ values vs. fractional nitrate use (Eq. (1)), and are reported ± the standard deviation of slope. Similarly, $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ values were determined from linear regressions of $\delta^{18}O$ onto corresponding $\delta^{15}N$ measurements of individual cultures.

Strain	$^{15}\varepsilon \pm 1\sigma$	$^{18}\varepsilon \pm 1\sigma$	$\Delta\delta^{18}O{:}\Delta\delta^{15}N \pm 1\sigma$
Pseudonitzchia hemii	6.4 ± 0.3	7.2 ± 0.5	1.12 ± 0.07
	4.8 ± 0.2	5.7 ± 0.2	1.19 ± 0.03
	5.3 ± 0.1	5.4 ± 0.2	1.02 ± 0.02
Phaedactylum tricornutum	8.0 ± 0.8	8.1 ± 0.6	1.02 ± 0.02
	5.5 ± 0.6	5.2 ± 0.9	0.91 ± 0.08
	4.6 ± 0.2	5.8 ± 0.9	1.22 ± 0.15
Chlorella vulgaris	8.6 ± 0.5	7.9 ± 0.6	0.91 ± 0.04
Chlorella pyrenoidosa	5.7 ± 0.2	6.9 ± 0.2	1.21 ± 0.08
	6.1 ± 0.8	6.4 ± 0.9	1.04 ± 0.06
Synechococcus sp. WH8102	3.9 ± 0.2	3.8 ± 0.1	0.95 ± 0.03
	4.2 ± 0.1	3.6 ± 0.1	0.85 ± 0.03
	3.5 ± 0.3	3.3 ± 0.0	0.95 ± 0.08
	3.8 ± 0.2	3.3 ± 0.1	0.88 ± 0.02
Synechococcus sp. WH7803 DC2	4.7 ± 0.2	5.5 ± 0.2	1.16 ± 0.06
	5.1 ± 0.1	6.0 ± 0.2	1.17 ± 0.04
	3.5 ± 0.1	2.9 ± 0.1	0.85 ± 0.01
Synechococcus sp. SNC1	2.2 ± 0.2	2.9 ± 0.3	1.29 ± 0.17
Alteromonas macleodii (Jul88)	1.0 ± 0.1	2.0 ± 0.1	2.06 ± 0.12
	0.4 ± 0.2	0.9 ± 0.3	1.89 ± 0.17
	0.6 ± 0.1	1.4 ± 0.2	2.19 ± 0.29
	0.9 ± 0.1	1.8 ± 0.0	1.99 ± 0.11

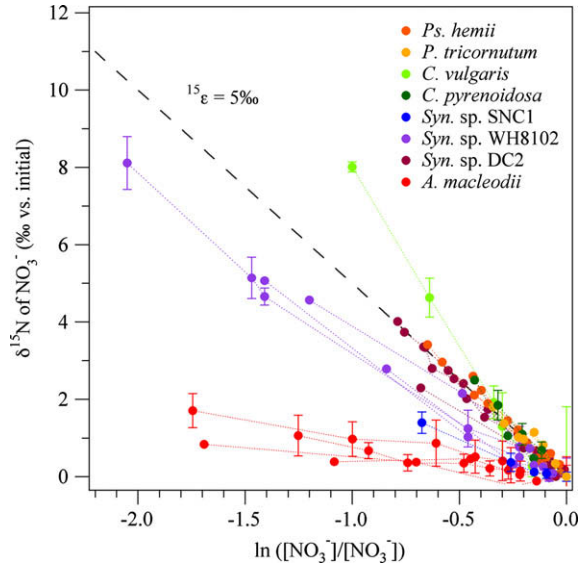

Fig. 2. Rayleigh plots of the change in the $\delta^{15}N$ of nitrate vs. the natural log of fractional nitrate consumption during its assimilation by mono-cultures of eukaryotic and prokaryotic unicellular phytoplankton. Standard deviations are provided for sample measurements that were replicated. The dashed line illustrates the trajectory for $^{15}\varepsilon = 5‰$.

apparently similar culture conditions (Needoba et al., 2003; Granger et al., 2004). And finally, there is no relationship between isotope effect amplitudes and variations in cell volume, within or among species (Needoba et al., 2003; Granger et al., 2004). If nitrate efflux were due to passive leakage, smaller cells, which have a larger ratio of cell surface area relative to cell volume, could show larger proportional nitrate efflux, and thus, diagnostically higher isotope effects, in analogy to the postulated dependence of carbon isotope fractionation on cell size during photosynthetic carbon assimilation (Popp et al., 1998). The lack of correlation to cell volume intimates that cells maintain internal nitrate homeostasis, by balancing internal nitrate depletion from enzymatic reduction and cellular efflux with nitrate uptake. Regardless of volume, cells ostensibly maintain comparable cellular efflux to gross uptake ratios, thus manifesting similar isotope effect amplitudes.

Coherence in isotope effect amplitudes emerges from studies that have demonstrated significant plasticity of the N isotope effects among cultures of diatoms and prymnesiophytes with respect to irradiance, wherein light-limited growth results in significantly larger isotope discrimination (Wada and Hattori, 1978; Needoba et al., 2004). At the origin of the irradiance-driven modulation of the isotope effect is a decrease in light-limited nitrate reductase activity (and growth rate) relative to nitrate uptake, manifested as an increase in cellular efflux, as well as by a substantial increase in internal nitrate concentrations in some diatom species (Needoba et al., 2004). Needoba et al. (2004) thus proposed that plasticity in the isotope effect amplitude could be conferred by vacuoles, which can compensate for imbalances in

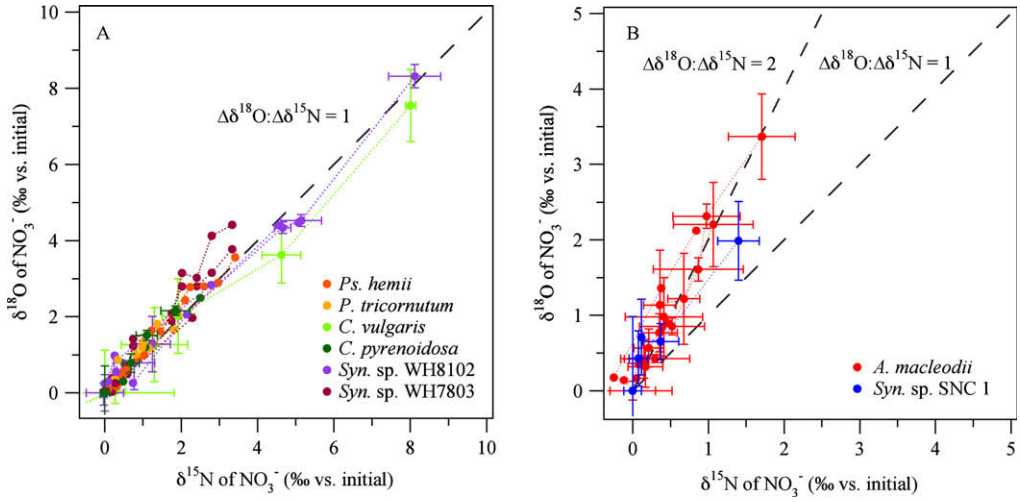

Fig. 3. The $\delta^{18}O$ of nitrate plotted over the corresponding $\delta^{15}N$ for (A) all experimental cultures save those of (B) *A. macleodii* and *Syn.* sp. SNC1 – shown on a magnified scale. Standard error bars were plotted for sample measurements that were replicated.

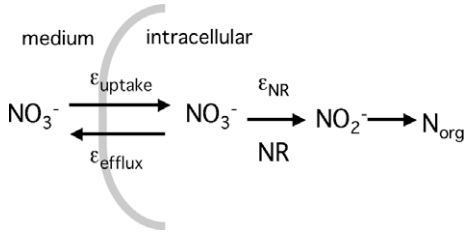

Fig. 4. Current view of nitrate isotope discrimination by unicellular algae (Shearer et al., 1991; Granger et al., 2004; Needoba et al., 2004). Nitrate reduction is the rate limiting step during cellular nitrate assimilation (Berges and Harrison, 1995), as well as the first irreversible step. Catalytic bond breakage at the site of nitrate reductase (NR) imparts significant isotope fractionation on internal nitrate, with enzymatic N isotope effects reportedly ranging from 15‰ to 25‰. The internal nitrate pool is thus isotopically enriched relative to external nitrate, showing a $\Delta\delta^{18}O:\Delta\ \delta^{15}N$ of 1. The enrichment of internal nitrate is propagated to the medium by cellular nitrate efflux. A small isotope effect imposed during transport and/or efflux is also plausible.

relative nitrate uptake to efflux in order to maintain cytosolic nitrate homeostasis. Implicitly, the lack of vacuoles in prokaryotes may impose a stricter balance between nitrate reduction and uptake in order to maintain optimal cytosolic nitrate concentrations, resulting in a constancy in the ratio of cellular efflux to gross nitrate uptake that could explain an apparently narrower range of isotope effect amplitudes seemingly manifested by prokaryotes.

4.2. The $\Delta\delta^{18}O:\Delta\delta^{15}N$ during nitrate assimilation

4.2.1. Comparison of eukaryotic and prokaryotic algae

Among the eukaryotes studied here, $^{18}\varepsilon$ appeared to be close to corresponding $^{15}\varepsilon$. Our present results and those from our previous study (Granger et al., 2004) thus confirm a $\Delta\delta^{18}O:\Delta\delta^{15}N$ of ~1 among all eukaryotic clades examined thus far. In this regard, the fresh water origins of *C. vulgaris* and *C. pyrenoidosa* are noteworthy, as these are the first freshwater eukaryotes for which coupled N and O isotope effects are reported, thus validating the $\Delta\delta^{18}O:\Delta\delta^{15}N$ of 1 as a benchmark for nitrate isotope studies in all aquatic systems, marine and terrestrial.

The fortuitous $\Delta\delta^{18}O:\Delta\delta^{15}N$ of ~1 during nitrate assimilation by eukaryotic algae most likely originates from isotope discrimination during enzymatic nitrate reduction, wherein nitrate reductase activity imparts equivalent O vs. N heavy isotope enrichments on nitrate (Fig 4; Granger et al., 2004). The similarity in $\Delta\delta^{18}O:\Delta\delta^{15}N$ among cultured species argues for similarity of the catalytic mechanism of assimilatory nitrate reductases among eukaryotic clades (Tcherkez and Farquhar, 2006).

Our observations also provide important confirmation that a $\Delta\delta^{18}O:\Delta\delta^{15}N$ of 1 extends to cyanobacterial assimilation. Cyanobacteria are an important group of primary producers in lacustrine, riverine, and marine environments, such that elucidation of the characteristic $\Delta\delta^{18}O:\Delta\delta^{15}N$ is important for environmental studies.

The uniformity of the cyanobacterial and eukaryotic $\Delta\delta^{18}O:\Delta\delta^{15}N$ relationships is also interesting in the context of the phylogenic relationships of nitrate reductase enzymes. Eukaryotic nitrate reductases are mono-nuclear molybdenum enzymes that share a common prokaryotic ancestor (Stolz and Basu, 2002). However, they bear characteristic structural differences from bacterial nitrate reductases, warranting their respective classification into different families of molybdo-enzymes (Table 2). Parenthetically, little is known about archeal nitrate reductases, yet these appear closely related to their functional bacterial counterparts based on sequence information (Stolz and Basu, 2002). In any case, in spite of distinguishing structural differences, both eukaryotic and bacterial assimilatory nitrate reductases, "eukNR" and "NAS," respectively – or at least the ferrodoxin-dependent assimilatory "NarB" cyanobacterial sub-clade of "NAS" (Stolz and Basu, 2002) – appear to impart nitrate N and O isotope effects that co-vary with a ratio of ~1. A $\Delta\delta^{18}O:\Delta\delta^{15}N$ relation-

Table 2
Molybdo-enzymes involved in biological nitrate reduction.

Nitrate reductase	Organisms	Function	Cellular location	Enzyme family	Phylogenic clade[a]	$\Delta\delta^{18}O:\Delta\delta^{15}N$
eukNR	Eukaryotes	Assimilatory	Cytoplasm	Sulfite oxidase	eukNR	1[b,d]
NAR	Prokaryotes	Respiratory	Membrane	DMSO reductase	NAR	1[c]
Nap	Prokaryotes	Redox balance	Periplasm	DMSO reductase	NAS	0.6[d]
NAS	Prokaryotes	Assimilatory	Cytosol	DMSO reductase	NAS	1[e]

[a] Stolz and Basu (2002).
[b] Granger et al. (2004), this study.
[c] Freshwater marine and α-preoteobacterial denitrifiers (Granger et al., 2008).
[d] "Nap" of *Rhodobacter sphaeroides* (Granger et al., 2008).
[e] This study for cyanobacteria, which have the ferrodoxin-dependent "NarB" sub-clade of the "NAS" clade.

ship of ~1 has also been shown to characterize bacterial denitrification (Granger et al., 2008) and is similarly posited to be imprinted on internal nitrate during bond breakage at the active site of the membrane-bound respiratory nitrate reductase, "NAR" (Table 2). Overall, the uniformity of the $\Delta\delta^{18}O:\Delta\delta^{15}N$ relationship among these nitrate reductases argues for close analogy in the mechanism of enzymatic catalysis of functionally and structurally distinct enzymes that are relatively distantly related.

4.2.2. The distinctive $\Delta\delta^{18}O:\Delta\delta^{15}N$ of *A. macleodii*

Given the posited dominance of nitrate reductase in imparting the N and O isotope effects during nitrate assimilation, nitrate reduction by the bacterial assimilatory "NAS" nitrate reductase could be at the origin of the $\Delta\delta^{18}O:\Delta\delta^{15}N$ of ~2 observed for *A. macleodii*. While plausible, such an enzymatic ratio would nonetheless appear unusual in the context the $\Delta\delta^{18}O:\Delta\delta^{15}N$ value of ~1 observed for nitrate assimilation by all other organisms studied thus far. In particular, this study confirms $\Delta\delta^{18}O:\Delta\delta^{15}N$ of ~1 among cyanobacteria, which, like *A. macleodii*, possess assimilatory nitrate reductases that group within the "NAS" clade. However, not all nitrate reductases conform to the same $\Delta\delta^{18}O:\Delta\delta^{15}N$: We previously observed that nitrate reduction effectuated by a prokaryotic periplasmic nitrate reductase, "Nap," a non-respiring, non-assimilating nitrate reductase involved in redox balancing, discriminates with a $\Delta\delta^{18}O:\Delta\delta^{15}N$ of ~0.6 (Table 2; Granger et al., 2008). "Nap" clusters as a sub-clade of the larger bacterial "NAS" clade (Table 2), which illustrates that phylogenetic relatedness does not invariably translate to homologous isotope discrimination among nitrate reductase enzymes. Moreover, the high variability among available "NAS" sequences (Stolz and Basu, 2002) implies that coupled N and O isotope effects among "NAS" enzymes could prove similarly heterogeneous. In this context, the distinct $\Delta\delta^{18}O:\Delta\delta^{15}N$ of 2 observed for *A. macleodii* might be due to its nitrate reductase.

Nevertheless, a more parsimonious explanation for the $\Delta\delta^{18}O:\Delta\delta^{15}N$ of 2 is the expression of a transport isotope effect at low amplitude of $^{15}\varepsilon$ (and $^{18}\varepsilon$). As noted earlier, the very low amplitude isotope fractionation by *A. macledii* suggests minimal expression of the nitrate reductase N and O isotope effects, due to minimal nitrate efflux countering gross uptake. In this case, an isotope effect of transport could contribute significantly to the organism-level isotope effect. In the absence of cellular nitrate efflux, transport would become the first irreversible step in nitrate assimilation; as such, transport would be the only isotope-sensitive step during assimilation. Parenthetically, any isotope effects associated with the aqueous diffusion of nitrate would *not* be evidenced by a change in the isotope composition of nitrate during growth of the cultures, because diffusion is not a rate determining step during nitrate assimilation: The gross diffusive flux of nitrate to the cell surface is orders of magnitude greater than the concomitant rate of transport by the cells at corresponding experimental nitrate concentrations (Pasciak and Gavis, 1974; Granger et al., 2008).

While isotope effects associated with transport are expected to be minor (Melander and Saunders, 1980), they would likely be greater for the O atoms than for the N atom of nitrate, because the oxygen atoms are subject to greater changes in their bonding environments during transport than is the N atom. For one, the three O atoms in nitrate form hydrogen bonds with surrounding water molecules (Tongraar et al., 2006), such that hydration and dehydration events that may be associated with cellular nitrate transport would impose direct changes in the bonding environment of the O atoms, which could translate to measurable differences in transport rates of the heavy vs. light O isotopologues of nitrate (Driesner et al., 2000; Bourg and Sposito, 2007). Similarly, the O atoms of nitrate may undergo transitory hydrogen bonding within nitrate transporters, which could further impart small but additive isotope effects among O isotopologues of nitrate. The more substantial discrimination anticipated for the oxygen isotopologues would entail a progressive decoupling of O vs. N isotope effects as the respective magnitudes of the organism-level $^{18}\varepsilon$ and $^{15}\varepsilon$ decrease, namely, as the proportional influence of transport to the organism-level $^{18}\varepsilon$ and $^{15}\varepsilon$ increases. Our observations for *A. macleodii* could be indicative of such a positive decoupling of the $\Delta\delta^{18}O:\Delta\delta^{15}N$ at lower magnitudes of $^{18}\varepsilon$ and $^{15}\varepsilon$, when cellular efflux is proportionally low relative to gross nitrate uptake.

In order to illustrate the contribution of putative N and O transport isotope effects to the organism-level N and O isotope effects based on Fig. 4, we propose an approximate expression that describes the nitrate isotope balance of a cell (François et al., 1993):

$$^{15}\varepsilon_{organsim} = {}^{15}\varepsilon_{up} + R({}^{15}\varepsilon_{NR} - {}^{15}\varepsilon_{ef}) \quad (3a)$$

$$^{18}\varepsilon_{organsim} = {}^{18}\varepsilon_{up} + R({}^{18}\varepsilon_{NR} - {}^{18}\varepsilon_{ef}) \quad (3b)$$

$^{15}\varepsilon_{organism}$ and $^{18}\varepsilon_{organism}$ are the isotope effects observed at the scale of the medium by the organisms. The terms ε_{up}, ε_{NR} and ε_{ef} represent the N or O isotope effects for nitrate uptake, enzymatic nitrate reduction by nitrate reductase, and nitrate efflux, respectively, while R is equal to the ratio of cellular nitrate efflux relative to gross nitrate uptake. When there is no cellular efflux ($R = 0$), $\varepsilon_{organism} = \varepsilon_{up}$. Conversely, given a reverse flow of nitrate out of the cell into the external pool ($0 < R < 1$), $\varepsilon_{organism}$ is proportionally sensitive to the relative amount of nitrate effluxed (R) and to its isotopic composition. In turn, the isotopic composition of effluxed nitrate is influenced largely by the enzymatic isotope effect, ε_{NR}, and possibly by isotope discrimination during efflux, ε_{ef}.

We solve for $^{15}\varepsilon_{organism}$ and $^{18}\varepsilon_{organism}$ as a function of the efflux to gross uptake ratio ($R = 0$–1) for four cases, each with a distinct combinations of assigned values for ε_{up}, ε_{NR} and ε_{ef} for N and O isotopes, respectively. The reductase N and O isotope effects are assumed to be equivalent to each other in all cases ($^{18}\varepsilon_{NR} = {}^{15}\varepsilon_{NR}$). Transport isotope effects are chosen from the observed range of isotope effect amplitudes observed for *A. macleodii*, where $^{15}\varepsilon_{up} = 0.4‰ - 1‰$ and $^{18}\varepsilon_{up} = 0.9‰ - 2‰$ (as per Table 1). Given no observational constraints on the magnitude of ε_{ef}, this parameter is assumed to be either nil or equivalent to its corresponding ε_{up}. Values of $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ are then calculated from the quotient of corresponding $^{18}\varepsilon_{organism}$ and $^{15}\varepsilon_{organism}$ derived for each case, and these are compared to the experimental data from this study as well as from our previous work (Fig. 5). The computed $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ trajectories vs. the corresponding $^{15}\varepsilon_{organism}$ indicate that the lower extent of isotope effects assigned to uptake, namely 0.4‰ and 0.9‰ for $^{15}\varepsilon_{up}$ and $^{18}\varepsilon_{up}$, respectively, best reproduce the positive departure of $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ at low isotope effect amplitude that is observed in the data. Higher assigned values of ε_{up} result in a less concave curve that asymptotes to the observed $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ of ~1 at higher values of $^{15}\varepsilon_{organism}$ (Fig. 5). Interestingly, ε_{ef} has little bearing on the $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ trajectory, by function of being insignificant relative to the value of ε_{NR}.

Overall, the above calculations illustrate that the $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ of ~2 observed for nitrate assimilation by *A. macleodii* may reveal minor O vs. N isotope effects associated with nitrate uptake, which are only manifested at low amplitude of the organism-level isotope effects. Similarly, the $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ of 1.3 for *Syn.* sp. SNC1 could also be consistent with the manifestation of transport isotope effects, given its relatively low organism-level $^{15}\varepsilon$ of 2‰ (Table 1; Fig. 5). In any case, the singular isotopic behaviour displayed by *A. macleodii* raises fundamental questions regarding the origin of the observed patterns, such that nitrate N and O isotope fractionation during nitrate assimilation by heterotrophic prokaryotes clearly merits further investigation.

4.3. Interpretation of the community N isotope effect in at the surface ocean

A hypothesis that emerges from our data is that prokaryotes, particularly heterotrophic prokaryotes, may express a lower mean range of N isotope effects than eukaryotic cells during nitrate assimilation. In the surface ocean, current estimates of $^{15}\varepsilon$ for nitrate assimilation appear confined to a narrower range than culture estimates, mostly falling between 4‰ and 10‰ (Wu et al., 1997; Sigman et al., 1999; Lourey et al., 2003; DiFiore et al., 2006). Such community isotope effects likely reflect the weighted average of various isotope effects imparted by

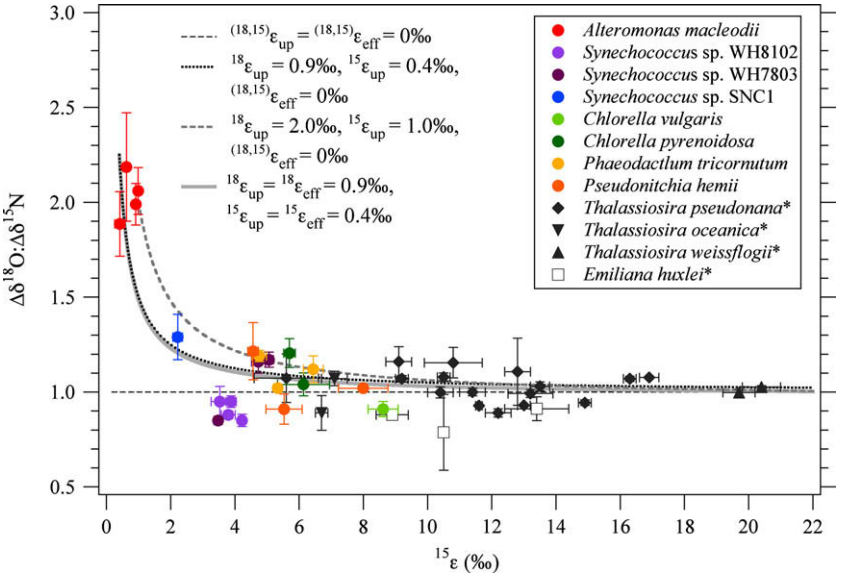

Fig. 5. $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ plotted against $^{15}\varepsilon$ for all experimental cultures. Data from a previous study, designated by asterisks, are also included. The error bars represent the standard error of the respective regression coefficients (Table 1). Four (4) simulations of $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ vs. $^{15}\varepsilon_{organism}$, are overlain on the experimental data, each computed from Eq. (3) with prescribed values for the O and N isotope effects for nitrate uptake ($^{(18,15)}\varepsilon_{up}$) and efflux ($^{(18,15)}\varepsilon_{ef}$). $^{18}\varepsilon_{NR} = {}^{15}\varepsilon_{NR} = 25‰$ in all four model cases.

the nitrate-utilizing species of the extant community (Karsh et al., 2003). Needoba (2004) followed the consumption of nitrate in on-deck bottle incubations of surface water collected in the subarctic Pacific, and observed larger N isotope discrimination in the accumulated particle product (i.e., particles trapped on a 0.7 μm pore-size filter) than in the nitrate substrate. Lower amplitude isotope discrimination by the bacterial size fraction that was not captured onto the filters was invoked as a possible cause of the apparent discrepancy between nitrate and particles. This hypothesis is consistent with the current observation that heterotrophic bacteria do not discriminate nitrate isotopes significantly. Thus, the meager nitrate isotope discrimination imparted by *A. macleodii* implies that nitrate use by heterotrophic bacteria could have a significant influence on the amplitude of the community nitrate N (and O) isotope effects at the surface ocean, countering more significant isotope discrimination due to nitrate use by larger plankton classes. The low amplitude of nitrate N isotope fractionation by heterotrophic bacteria could prove useful as a diagnostic of nitrate use by bacterioplankton. Moreover, different isotope effect amplitudes by different plankton size fractions could also prove important in partitioning of N isotopes among fast-sinking organisms such a diatoms and coccolithophores vs. prokaryotic biomass that has a longer residence time in mixed layer. This has implications, among others, for paleo-reconstruction of surface N budgets from sedimentary N isotopes, which relies on a firm understanding of the determinants of the amplitude of N isotope discrimination associated with N assimilation at the surface ocean, and on the transfer of this signal to the sediment. Defining the contribution of plankton groups to the community isotope effect and to the community N isotope budget will undoubtedly yield important insight into N biogeochemistry.

4.4. The $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ in the surface ocean

While the contribution of heterotrophic bacteria to the amplitude of the community N and O isotope effects may prove significant, bacterial nitrate assimilation would apparently not modify the community $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ appreciably. Granger et al. (2004) initially proposed that given an invariant $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ of ~1 for nitrate assimilation, any observed deviation from a $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ of 1 in surface ocean samples could reflect co-occurring N transformations. Wankel et al. (2007) subsequently reported significant positive deviations of the $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ from 1 in surface waters of the California upwelling, from which the authors estimated a large contribution of *in situ* regeneration and nitrification to the surface nitrate pool. In light of our current observations, could the positive deviations in observed by Wankel et al. (2007) have reflected some contribution of nitrate assimilation by heterotrophic prokaryotes? Not likely, given that deviations from a $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ of 1 would only become significant at very low amplitudes of the community isotope effects (i.e., $^{15}\varepsilon \leqslant 2‰$), as per Fig. 5. The isotope effect observed in the surface ocean is generally $\geqslant 5‰$, at which amplitudes transport isotope effects would not alter the observed $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ detectably. Thus, transport isotope effects do not explain the $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ deviations observed by Wankel et al. (2007). Nonetheless, validating the proposed physiological mechanism in imparting the distinct $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ of *A. macleodii* and investigating the pervasiveness of this alternate $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ among bacterial strains will be key to gauging its importance in the environment.

ACKNOWLEDGMENTS

We thank Y. Wang for assistance with isotope analyses, and B. B. Ward for valuable insight. A. Marchetti providing *Ps. hemii* diatom isolate. Comments by two anonymous reviewers helped improve the text significantly. This work was funded by a seed grant from CEBIC (Center for Environmental BioInorganic Chemistry), a University of British Columbia Graduate Fellowship awarded to J. Granger, and U.S. NSF grant OCE-0447570 (CAREER) to D. Sigman.

REFERENCES

Allen A. E., Howard-Jones M. H., Booth M. G., Frischer M. E., Verity P. G., Bronk D. A. and Sanderson M. P. (2002) Importance of heterotrophic bacterial assimilation of ammonium and nitrate in the Barents Sea during summer. *J. Mar. Syst.* **38**, 93–108.

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 Res. A–Oceanogr. Res. Pap.* **35**, 535–554.

Altabet M. A. (2001) Nitrogen isotopic evidence for micronutrient control of fractional NO_3^-utilization in the equatorial Pacific. *Limnol. Oceanogr.* **46**, 368–380.

Berges J. A. and Harrison P. J. (1995) Nitrate reductase-activity quantitatively predicts the rate of nitrate incorporation under steady-state light limitation – a revised assay and characterization of the enzyme in 3 species of marine phytoplankton. *Limnol. Oceanogr.* **40**, 82–93.

Böhlke J. K., Mroczkowski S. J. and Coplen T. B. (2003) Oxygen isotopes in nitrate: new reference materials for O-18: O-17: O-16 measurements and observations on nitrate–water equilibration. *Rapid Commun. Mass Spectrom.* **17**, 1835–1846.

Böhlke J. K., Smith R. L. and Hannon J. E. (2007) Isotopic analysis of N and O in nitrite and nitrate by sequential selective bacterial reduction to N_2O. *Anal. Chem.* **79**, 5888–5895.

Bourg I. C. and Sposito G. (2007) Molecular dynamics simulations of kinetic isotope fractionation during the diffusion of ionic species in liquid water. *Geochim. Cosmochim. Acta* **73**, 5583–5598.

Braman R. S. and Hendrix S. A. (1989) Nanogram nitrite and nitrate determination in environmental and biological materials by V(III) reduction with chemiluminescence detection. *Anal. Chem.* **61**, 2715–2718.

Brandes J. A. and Devol A. H. (2002) A global marine-fixed nitrogen isotopic budget: implications for holocene nitrogen cycling. *Global Biogeochem. Cycles* **16**.

Brandes J. A., Devol A. H., Yoshinari T., Jayakumar D. A. and Naqvi S. W. A. (1998) Isotopic composition of nitrate in the central Arabian Sea and eastern tropical North Pacific: a tracer for mixing and nitrogen cycles. *Limnol. Oceanogr.* **43**, 1680–1689.

Casciotti K. L., Bohlke J. K., McIlvin M. R., Mroczkowski S. J. and Hannon J. E. (2007) Oxygen isotopes in nitrite: analysis, calibration, and equilibration. *Anal. Chem.* **79**, 2427–2436.

Casciotti K. L., Sigman D. M., Hastings M. G., Bohlke J. K. and Hilkert A. (2002) Measurement of the oxygen isotopic composition of nitrate in seawater and freshwater using the denitrifier method. *Anal. Chem.* **74**, 4905–4912.

Cline J. D. and Kaplan I. R. (1975) Isotopic fractionation of dissolved nitrate during denitrification in the Eastern Tropical North Pacific Ocean. *Mar. Chem.* **3**, 271–299.

Deutsch C., Sigman D. M., Thunell R. C., Meckler A. N. and Haug G. H. (2004) Isotopic constraints on glacial/interglacial changes in the oceanic nitrogen budget. *Global Biogeochem. Cycles* **18**.

DiFiore P. J., Sigman D. M., Trull T. W., Lourey M. J., Karsh K., Cane G. and Ho R. (2006) Nitrogen isotope constraints on subantarctic biogeochemistry. *J. Geophys. Res.-Oceans* **111**.

Driesner T., Ha T. K. and Seward T. M. (2000) Oxygen and hydrogen isotope fractionation by hydration complexes of Li^+, Na^+, K^+, Mg^{2+}, F^-, Cl^-, and Br^-: a theoretical study. *Geochim. Cosmochim. Acta* **64**, 3007–3033.

François R., Altabet M. A., Yu E. F., Sigman D. M., Bacon M. P., Frank M., Bohrmann G., Bareille G. and Labeyrie L. D. (1997) Contribution of Southern Ocean surface-water stratification to low atmospheric CO_2 concentrations during the last glacial period. *Nature* **389**, 929–935.

François R., Bacon M. P., Altabet M. A. and Labeyrie L. D. (1993) Glacial interglacial changes in sediment rain rate in the sw Indian sector of sub-Antarctic waters as recorded by Th-230, Pa-231, U, and delta-N-15. *Paleoceanography* **8**, 611–629.

Gonfiantini R., Stichler W. and Rosanski K. (1995) *Standards and Intercomparison Materials Distributed by the IAEA for Stable Isotope Measurements*. International Atomic Energy Agency, Vienna.

Granger J., Sigman D. M., Lehmann M. F. and Tortell P. D. (2008) Nitrogen and oxygen isotope fractionation during dissimilatory nitrate reduction by denitrifying bacteria. *Limnol. Oceanogr.* **53**, 2533–2545.

Granger J., Sigman D. M., Needoba J. A. and Harrison P. J. (2004) Coupled nitrogen and oxygen isotope fractionation of nitrate during assimilation by cultures of marine phytoplankton. *Limnol. Oceanogr.* **49**, 1763–1773.

Karsh K. L., Trull T. W., Lourey A. J. and Sigman D. M. (2003) Relationship of nitrogen isotope fractionation to phytoplankton size and iron availability during the Southern Ocean Iron RElease Experiment (SOIREE). *Limnol. Oceanogr.* **48**, 1058–1068.

Keller M. D., Bellows W. K. and Guillard R. R. L. (1988) Microwave treatment for sterilization of phytoplankton culture media. *J. Exp. Mar. Biol. Ecol.* **117**, 279–283.

Knapp A. N., Sigman D. M. and Lipschultz F. (2005) N isotopic composition of dissolved organic nitrogen and nitrate at the Bermuda Atlantic time-series study site. *Global Biogeochem. Cycles* **19**, GB1018.

Ledgard S. F., Woo K. C. and Bergersen F. J. (1985) Isotopic fractionation during reduction of nitrate to nitrite by extracts of spinach leaves. *Aust. J. Plant Physiol.* **12**, 631–640.

Lehmann M. F., Sigman D. M. and Berelson W. M. (2004) Coupling the N-15/N-14 and O-18/O-16 of nitrate as a constraint on benthic nitrogen cycling. *Mar. Chem.* **88**, 1–20.

Lehmann M. F., Sigman D. M., McCorkle D. C., Brunelle B. G., Hoffmann S., Kienast M., Cane G. and Clement J. (2005) Origin of the deep Bering Sea nitrate deficit: constraints from the nitrogen and oxygen isotopic composition of water column nitrate and benthic nitrate fluxes. *Global Biogeochem. Cycles* **19**, GB4005.

Liu K. K., Su M. J., Hsueh C. R. and Gong G. C. (1996) The nitrogen isotopic composition of nitrate in the Kuroshio Water northeast of Taiwan: evidence for nitrogen fixation as a source of isotopically light nitrate. *Mar. Chem.* **54**, 273–292.

Lourey M. J., Trull T. W. and Sigman D. M. (2003) Sensitivity of delta N-15 of nitrate, surface suspended and deep sinking particulate nitrogen to seasonal nitrate depletion in the Southern Ocean. *Global Biogeochem. Cycles* **17**.

Mariotti A., Germon J. C., Hubert P., Kaiser P., Letolle R., Tardieux A. and Tardieux P. (1981) Experimental determination of nitrogen kinetic isotope fractionation: some principles; illustration for the denitrification and nitrification processes. *Plant Soil* **62**, 413–430.

McIlvin M. R. and Altabet M. A. (2005) Chemical conversion of nitrate and nitrite to nitrous oxide for nitrogen and oxygen isotopic analysis in freshwater and seawater. *Anal. Chem.* **77**, 5589–5595.

Melander L. and Saunders W. H. J. (1980) *Reaction Rates of Isotopic Molecules*. John Wiley, New York.

Montoya J. P. and McCarthy J. J. (1995) Isotopic fractionation during nitrate uptake by marine phytoplankton grown in continuous culture. *J. Plankton Res.* **17**, 439–464.

Needoba J. (2004) *Nitrogen Isotope Fractionation by Four Groups of Marine Algae During Growth on Nitrate*. University of British Columbia.

Needoba J. A. and Harrison P. J. (2004) Influence of low light and a light: dark cycle on NO_3^- uptake, intracellular NO_3^-, and nitrogen isotope fractionation by marine phytoplankton. *J. Phycol.* **40**, 505–516.

Needoba J. A., Sigman D. M. and Harrison P. J. (2004) The mechanism of isotope fractionation during algal nitrate assimilation as illuminated by the N-15/N-14 of intracellular nitrate. *J. Phycol.* **40**, 517–522.

Needoba J. A., Waser N. A., Harrison P. J. and Calvert S. E. (2003) Nitrogen isotope fractionation in 12 species of marine phytoplankton during growth on nitrate. *Mar. Ecol.: Prog. Ser.* **255**, 81–91.

Pasciak W. J. and Gavis J. (1974) Transport limitation of nutrient uptake in phytoplankton. *Limnol. Oceanogr.* **19**, 881–898.

Pennock J. R., Velinsky D. J., Sharp J. H., Ludlam J. and Fogel M. L. (1996) Isotope fractionation of ammonium and nitrate during their uptake by *Skeletonema costatum*.: implications for the $\delta^{15}N$ dynamics under bloom conditions. *Limnol. Oceanogr.* **41**, 451–459.

Popp B. N., Laws E. A., Bidigare R. R., Dore J. E., Hanson K. L. and Wakeham S. G. (1998) Effect of phytoplankton cell geometry on carbon isotopic fractionation. *Geochim. Cosmochim. Acta* **62**, 69–77.

Price N. M., Harrison G. I., Herring J. G., Hudson R. J., Nirel P. M. V., Palenik B. and Morel F. M. M. (1988/1989) Preparation and chemistry of the artificial algal culture medium Aquil. *Biol. Oceanogr.* **6**, 443–461.

Robinson R. S., Sigman D. M., DiFiore P. J., Rohde M. M., Mashiotta T. A. and Lea D. W. (2005) Diatom-bound N-15/N-14: New support for enhanced nutrient consumption in the ice age subantarctic. *Paleoceanography* **20**.

Schmidt H. L. and Medina R. (1991) Possibilities and scope of the double isotope effect method in the elucidation of mechanisms of enzyme-catalyzed reactions. *Isotopenpraxis* **27**, 1–4.

Shearer G., Schneider J. D. and Kohl D. H. (1991) Separating the efflux and influx components of net nitrate uptake by *Synechococcus*-R2 under steady-state conditions. *J. General Microbiol.* **137**, 1179–1184.

Sigman D. M., Altabet M. A., François R., McCorkle D. C. and Fischer G. (1999) The $\delta^{15}N$ of nitrate in the Southern Ocean:

consumption of nitrate in surface waters. *Global Biogeochem. Cycles* **13**, 1149–1166.

Sigman D. M., Casciotti K. L., Andreani M., Barford C., Galanter M. and Bohlke J. K. (2001) A bacterial method for the nitrogen isotopic analysis of nitrate in seawater and freshwater. *Anal. Chem.* **73**, 4145–4153.

Sigman D. M., Granger J., DiFiore P. J., Lehmann M. M., Ho R., Cane G. and van Geen A. (2005) Coupled nitrogen and oxygen isotope measurements of nitrate along the eastern North Pacific margin. *Global Biogeochem. Cycles* **19**.

Sigman D. M., Robinson R., Knapp A. N., van Geen A., McCorkle D. C., Brandes J. A. and Thunell R. C. (2003) Distinguishing between water column and sedimentary denitrification in the Santa Barbara Basin using the stable isotopes of nitrate. *Geochem. Geophys. Geosyst.* **4**.

Silva S. R., Kendall C., Wilkinson D. H., Ziegler A. C., Chang C. C. Y. and Avanzino R. J. (2000) A new method for collection of nitrate from fresh water and the analysis of nitrogen and oxygen isotope ratios. *J. Hydrol.* **228**, 22–36.

Stolz J. F. and Basu P. (2002) Evolution of nitrate reductase: molecular and structural variations on a common function. *Chembiochem* **3**, 198–206.

Sutka R. L., Ostrom N. E., Ostrom P. H. and Phanikumar M. S. (2004) Stable nitrogen isotope dynamics of dissolved nitrate in a transect from the North Pacific Subtropical Gyre to the Eastern Tropical North Pacific. *Geochim. Cosmochim. Acta* **68**, 517–527.

Tcherkez G. and Farquhar G. D. (2006) Isotopic fractionation by plant nitrate reductase, twenty years later. *Funct. Plant Biol.* **33**, 531–537.

Tongraar A., Tangkawanwanit P. and Rode B. M. (2006) A combined QM/MM molecular dynamics simulations study of nitrate anion (NO_3^-) in aqueous solution. *J. Phys. Chem. A* **110**, 12918–12926.

Voss M., Altabet M. A. and vonBodungen B. (1996) Delta N-15 in sedimenting particles as indicator of euphotic-zone processes. *Deep-Sea Res. I: Oceanogr. Res. Pap.* **43**, 33–47.

Voss M., Dippner J. W. and Montoya J. P. (2001) Nitrogen isotope patterns in the oxygen-deficient waters of the Eastern Tropical North Pacific Ocean. *Deep-Sea Res. I: Oceanogr. Res. Pap.* **48**, 1905–1921.

Wada E. and Hattori A. (1978) Nitrogen isotope effects in the assimilation of inorganic nitrogenous compounds. *Geomicrobiol. J.* **1**, 85–101.

Wankel S. D., Kendall C., Pennington J. T., Chavez F. P. and Paytan A. (2007) Nitrification in the euphotic zone as evidenced by nitrate dual isotopic composition: Observations from Monterey Bay, California. *Global Biogeochem. Cycles* **21**.

Waser N. A., Yin K. D., Yu Z. M., Tada K., Harrison P. J., Turpin D. H. and Calvert S. E. (1998) Nitrogen isotope fractionation during nitrate, ammonium and urea uptake by marine diatoms and coccolithophores under various conditions of N availability. *Mar. Ecol.: Prog. Ser.* **169**, 29–41.

Wu J., Calvert S. E. and Wong C. S. (1997) Nitrogen isotope variations in the subarctic Pacific northeast Pacific: relationships to nitrate utilization and trophic structure. *Deep-Sea Res. I* **44**, 287–314.

Associate editor: Jay A. Brandes