

## Coupled nitrogen and oxygen isotope fractionation of nitrate during assimilation by cultures of marine phytoplankton

Julie Granger<sup>1</sup>

Department of Earth and Ocean Sciences, University of British Columbia, 6270 University Boulevard, Vancouver, British Columbia V6T 1Z4, Canada

Daniel M. Sigman

Department of Geosciences, Princeton University, Guyot Hall, Princeton, New Jersey 08544

Joseph A. Needoba and Paul J. Harrison<sup>2</sup>

Department of Earth and Ocean Sciences, University of British Columbia, 6270 University Boulevard, Vancouver, British Columbia V6T 1Z4, Canada

### Abstract

We report the first measurements of coupled nitrogen (N) and oxygen (O) isotopic variations of nitrate ( $\text{NO}_3^-$ ) during its assimilation by laboratory cultures of marine phytoplankton and derive the N and O kinetic isotope effects for nitrate assimilation by three species of diatoms (*Thalassiosira weissflogii*, *Thalassiosira oceanica*, and *Thalassiosira pseudonana*) and a coccolithophorid (*Emiliana huxleyi*). Large interspecies and intraspecies variations in the N isotope effects were observed. The O isotope effect associated with nitrate consumption was consistently close to the N isotope effect, such that the  $^{18}\text{O}/^{16}\text{O}$  and  $^{15}\text{N}/^{14}\text{N}$  of nitrate varied in a ratio of  $\sim 1:1$ , regardless of species or of the magnitude of the isotope effect. In addition, the  $^{18}\text{O}/^{16}\text{O}$  and  $^{15}\text{N}/^{14}\text{N}$  of internal nitrate of *T. weissflogii* grown under various environmental conditions were elevated relative to the medium nitrate by a proportion of  $\sim 1:1$ . These findings are consistent with a nitrate isotopic fractionation mechanism that involves nitrate reduction as the chief fractionating step. The observed N:O isotopic coupling during nitrate assimilation suggests that combined N and O isotopic measurements of water column nitrate can provide new constraints on the ocean N cycle.

Stable isotopes are a common tool in studies of the N cycle, both in the ocean and on land. Variations in the  $^{15}\text{N}/^{14}\text{N}$  ratio of inorganic nitrogen pools can provide an integrative picture of the N cycle, from which physical, chemical, and biochemical fluxes can be inferred. Environmental variations in  $^{15}\text{N}/^{14}\text{N}$  typically occur because, in most chemical and biochemical transformations, the rate of reaction of  $^{15}\text{N}$ -bearing substrate is slightly lower than that for the same substrate bearing  $^{14}\text{N}$ . The extent to which a biological N transformation fractionates between  $^{14}\text{N}$  and  $^{15}\text{N}$  is given by the isotope effect,  $^{15}\epsilon$ . In the case of a unidirectional reaction,  $^{15}\epsilon$  is referred to as the kinetic isotope effect and is a function of the ratio of the reaction rates ( $k$ ) for the molecules containing the two isotopes:  $^{15}\epsilon(\text{‰}) = (k^{14}/k^{15} - 1) \times 1000$ .

Owing to isotope fractionation during nitrate assimilation, the  $^{15}\text{N}/^{14}\text{N}$  of nitrate is observed to increase as it is consumed

in the surface ocean (Sigman et al. 1997; Wu et al. 1997; Sigman et al. 1999a). This isotopic change in nitrate is communicated by assimilation into phytoplankton biomass, resulting in meridional gradients in the  $^{15}\text{N}/^{14}\text{N}$  of sinking particulate N and surface sedimentary N in the Equatorial Pacific and in the Southern Ocean that are correlated with nitrate use (and thus anticorrelated with surface nitrate concentration; François et al. 1992; Altabet and François 1994; Farrell et al. 1995; François et al. 1997). Thus, down-core changes in the  $^{15}\text{N}/^{14}\text{N}$  of sediment and sedimentary fractions have been interpreted as reflecting past changes in the degree of nitrate consumption by phytoplankton in the surface ocean (François et al. 1992; Altabet and François 1994; Farrell et al. 1995; François et al. 1997; Sigman et al. 1999b). The isotopic signal of nitrate assimilation can also be used to study aspects of the modern ocean N cycle, for instance, the source of nitrate to surface waters in nutrient-rich regions (Sigman et al. 1999a).

The isotope effect is a key parameter linking nitrate assimilation to the  $^{15}\text{N}/^{14}\text{N}$  of nitrate and particulate N. The  $^{15}\text{N}/^{14}\text{N}$  of nitrate in the upper ocean typically suggests an isotope effect of 5–10‰ for nitrate assimilation, with most estimates closer to 5‰ (Wada 1980; Wu et al. 1997; Sigman et al. 1999a; Altabet 2001). In contrast, estimates of  $^{15}\epsilon$  derived from laboratory studies of marine phytoplankton show a wide range of variation, from 0‰ to 20‰ (Wada and Hattori 1978; Montoya and McCarthy 1995; Waser et al. 1998a; Needoba et al. 2003; this study). The observed variation in  $^{15}\epsilon$  among and within cultured phytoplankton species is not

<sup>1</sup> Corresponding author (jgranger@eos.ubc.ca).

<sup>2</sup> Present address: Atmospheric, Marine, and Coastal Environmental Program, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong SAR.

### Acknowledgments

We thank Greg Cane and Ruby Ho for technical assistance, Philippe Tortell for comments on the manuscript, and Frederico Zahariev for assistance with chemical computations. Comments by two anonymous reviewers helped to improve the manuscript. This work was funded by the Natural Sciences and Engineering Research Council of Canada (PGS-B), U.S. NSF grants OCE-9981479 and OCE-0081686, and the Department of Earth and Ocean Sciences at the University of British Columbia.

understood, and environmental controls on  $^{15}\epsilon$  are undefined, largely because the N isotope fractionation mechanism for nitrate assimilation remains uncertain. These unknowns limit the utility of the N isotopes in the modern ocean and in the effort to reconstruct past ocean conditions.

Previously, nitrate  $^{15}\text{N}/^{14}\text{N}$  in seawater has been measured by reduction to ammonia, followed by extraction of the ammonia by distillation (Cline and Kaplan 1975) or diffusion (Sigman et al. 1997), reaction to  $\text{N}_2$  gas (typically by combustion), and mass spectrometric analysis of the  $\text{N}_2$ . Here, we take advantage of a recently developed method for nitrate isotopic analysis that uses denitrifying bacteria to convert nitrate (and nitrite) to nitrous oxide ( $\text{N}_2\text{O}$ ), followed by isotopic analysis of the  $\text{N}_2\text{O}$  (Sigman et al. 2001; Casciotti et al. 2002). This "denitrifier" method for seawater nitrate isotope analysis has advantages relative to the ammonium-based methods that are critical to culture studies of marine phytoplankton. First, a roughly 100-fold reduction in sample size requirement allows for cultures of small to moderate volume. Second, a lack of cross-contamination by dissolved organic N or ammonium and a reduction in reagent blank are both critical for culture studies and allow for the reproducible isotopic analysis of samples with as little as  $0.5 \mu\text{mol L}^{-1}$  nitrate. Finally, and most central to this study, the denitrifier method is the first to allow O isotope analysis of nitrate in a saline solution such as seawater (Casciotti et al. 2002).

We report here a culture study of N and O isotope fractionation of nitrate during its assimilation by marine phytoplankton. To our knowledge, this study represents the first culture-based effort to characterize the relationship between the N and O isotopes of nitrate during an important N transformation. Our measurements of coupled N and O isotope fractionation provide new insight into the mechanism underlying isotopic fractionation during nitrate assimilation by marine phytoplankton. This has significance for the biological chemistry of nitrate assimilation, and for paleoceanographic studies, in which assumptions must be made about the isotope effect of nitrate assimilation in the past. Moreover, it provides a basis for use of the coupled N and O isotopes of nitrate to study modern ocean N cycling (Sigman et al. 2003; Lehmann et al. 2004).

## Methods

Experimental algal strains, *Thalassiosira weissflogii* (actin), *Thalassiosira oceanica*, *Thalassiosira pseudonana* (3H), and *Emiliana huxleyi*, were grown in semicontinuous batch cultures in the artificial seawater medium Aquil (Price et al. 1988/1989) at  $20^\circ\text{C}$  under continuous saturating light at  $150 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ . The artificial seawater mixture was amended with  $100 \mu\text{mol L}^{-1}$  silicate,  $10 \mu\text{mol L}^{-1}$  phosphate, and between 50 and  $150 \mu\text{mol L}^{-1}$  nitrate. It was then passed through Chelex 100 resin to remove contaminating trace metals (Price et al. 1988/1989). Metal-clean artificial seawater was stored in acid-washed polycarbonate bottles and sterilized by microwaving (Keller et al. 1988). Filter-sterilized f/2 vitamins were added to sterile media, as well as filter-sterilized Aquil trace metals chelated with  $100 \mu\text{mol}$

$\text{L}^{-1}$  ethylenediaminetetraacetic acid (EDTA; Price et al. 1988/1989). Cells were acclimated to respective media for two culture transfers (ca. eight generations) and inoculated in 500-ml polycarbonate media bottles.

Our focal interest was in the variations of nitrate N and O isotopic fractionation, both among and within species. Two of the experimental strains were thus subjected to a variety of culture conditions in an attempt to cause changes in the isotope effect manifested by a specific strain. In cultures of *T. weissflogii* and *T. oceanica*, iron concentrations were modulated to obtain iron-limited growth rates of the strains. Total Fe concentrations in incremental treatments were  $1 \mu\text{mol L}^{-1}$ ,  $100 \text{ nmol L}^{-1}$ ,  $45 \text{ nmol L}^{-1}$ , and  $12 \text{ nmol L}^{-1}$ , corresponding to approximate free ferric iron concentrations (expressed as  $\text{pFe} = -\log \text{Fe}^{3+}$ ) of 19, 20, 20.5, and 21, respectively (calculated with MINEQL; Westall et al. 1976). A set of experiments with *T. weissflogii* at various iron concentrations was also conducted in stirred culture vessels to compare with unstirred cultures. Finally, N and O isotopic fractionation by *T. weissflogii* was monitored in short-term nitrate uptake experiments. In these experiments, cells preconditioned in pFe19 or pFe20 medium were harvested in late exponential growth. Whole cultures (500 ml) were filtered onto acid-washed,  $5\text{-}\mu\text{m}$  pore-size polycarbonate filter and resuspended in 500 ml of fresh medium (of the same iron concentration). Subsamples of culture filtrate were then collected at short (ca. bihourly) time intervals.

Cell cultures were subsampled throughout exponential growth. Growth was monitored by cell counts on a Z series Coulter counter. For particulate N isotopic measurements, 20 ml of cell culture were gently filtered onto a precombusted AE glass-fiber filter. Filters were then dried at  $60^\circ\text{C}$ , pelleted into tin capsules, and sent for N isotopic analysis to David Harris at the Stable Isotope Facility, University of California, Davis. Isotope ratios of the particulate N on the filters were determined by continuous flow combustion/isotope mass spectrometry using a Europa ANCA elemental analyzer on-line with a Europa Hydra 20/20 mass spectrometer.

Intracellular nitrate was collected for isotopic comparison with the medium in four *T. weissflogii* cultures grown under conditions similar to those described above (described in detail by Needoba et al. in press). Briefly, cells were grown in semicontinuous batch cultures in artificial seawater with  $200 \mu\text{mol L}^{-1}$  nitrate. Cultures were initiated in filter-sterilized medium and incubated at  $18^\circ\text{C}$  under saturating or subsaturating light levels, and some cultures were also grown in iron-deplete medium. Exponentially growing cells (300–500 ml) were harvested onto a 47-mm precombusted GF/F filter, washed with 3% NaCl to remove remnant extracellular nitrate, and boiled in water to extract intracellular nitrate (Thorensen et al. 1982). Nitrate concentrations were then measured as described below, and the N and O isotopic compositions of internal nitrate were then determined with the denitrifier method (Sigman et al. 2001; Casciotti et al. 2002). The nitrate concentrations and N isotope data alone are reported by Needoba et al. (in press).

For N and O isotope analysis of nitrate, filtrate of exponentially growing cultures was collected in acid-washed 30-ml polypropylene bottles and immediately frozen until analysis. Nitrate concentrations of thawed samples were

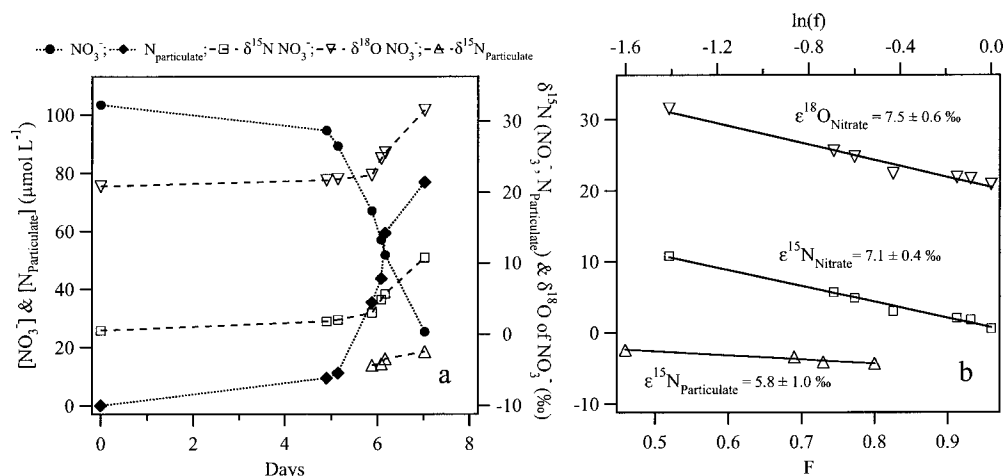


Fig. 1. Nitrate assimilation by *T. pseudonana* grown in batch culture in iron-replete medium (pFe19). (a) Disappearance of nitrate from the growth medium and the concomitant accumulation of cellular N over time. Also shown are the respective increases in nitrate  $\delta^{15}\text{N}$  and  $\delta^{18}\text{O}$ , as well as particulate N  $\delta^{15}\text{N}$  as nitrate concentrations diminish. (b) Rayleigh linearization of nitrate  $\delta^{15}\text{N}$  and  $\delta^{18}\text{O}$  over the  $\ln f$  (Eq. 3), and of particulate N  $\delta^{15}\text{N}$  (the accumulated product) over  $F$  (Eq. 4). The slopes of individual linear regressions correspond to the isotope effect,  $\varepsilon$  ( $\pm$ SE).

measured by conversion to NO (nitric oxide) followed by chemiluminescence detection (Braman and Hendrix 1989). Nitrite was also measured in this manner, although none was detected in any of the experimental samples (detection limit  $\sim 0.1 \mu\text{mol L}^{-1}$  nitrite). In the samples of growth medium and extracted intracellular pools, the  $^{15}\text{N}/^{14}\text{N}$  and  $^{18}\text{O}/^{16}\text{O}$  of nitrate were determined following the denitrifier method (Sigman et al. 2001; Casciotti et al. 2002). Isotope ratios are reported using delta ( $\delta$ ) notation in units of per mil (‰):

$$\delta^{15}\text{N}_{\text{sample}} = \left[ \frac{(^{15}\text{N}/^{14}\text{N})_{\text{sample}}}{(^{15}\text{N}/^{14}\text{N})_{\text{reference}}} - 1 \right] \times 1000 \quad (1)$$

$$\delta^{18}\text{O}_{\text{sample}} = \left[ \frac{(^{18}\text{O}/^{16}\text{O})_{\text{sample}}}{(^{18}\text{O}/^{16}\text{O})_{\text{reference}}} - 1 \right] \times 1000 \quad (2)$$

where the  $^{15}\text{N}/^{14}\text{N}$  reference is  $\text{N}_2$  in air and the  $^{18}\text{O}/^{16}\text{O}$  reference is Vienna standard mean ocean water (VSMOW). Referencing to air and VSMOW was through comparison to the international potassium nitrate reference material IAEA-N3, with an assigned  $\delta^{15}\text{N}$  of +4.7‰ (Gonfiantini et al. 1995) and reported  $\delta^{18}\text{O}$  of +22.7 to +25.6‰ (Revesz et al. 1997; Silva et al. 2000; Böhlke et al. 2003; Lehmann et al. 2003). We adopted a  $\delta^{18}\text{O}$  of 22.7‰, but without consequence, since only isotope ratio differences are used in this study. The N and O isotopic ratios represent the mean of any replicate measurements; the N and O isotopic ratio measurements of roughly 50% of the samples were duplicated within a day's batch of analyses, and individual samples were analyzed in one to three of the individual batches of analysis. Reproducibility of the replicates was generally consistent with previously reported analysis standard deviations of 0.2‰ for  $\delta^{15}\text{N}$  and 0.5‰ for  $\delta^{18}\text{O}$ .

N and O isotopic ratios for individual experiments were fitted to the Rayleigh isotope fractionation model to determine the isotope effect (Mariotti et al. 1981). Nitrate N and

O isotopic measurements were modeled according to the following Rayleigh linearization:

$$\delta^{15}\text{N}(\text{or } \delta^{18}\text{O})_{\text{reactant}} = \delta^{15}\text{N}(\text{or } \delta^{18}\text{O})_{\text{initial}} - \varepsilon(\ln f) \quad (3)$$

where nitrate is the reactant and  $f$  is the fraction of the initial nitrate pool that remains. In some experiments,  $\varepsilon$  was also determined from the  $^{15}\text{N}/^{14}\text{N}$  of accumulated particulate N in the culture bottles, using the Rayleigh integrated product equation (Mariotti et al. 1981)

$$\delta^{15}\text{N}_{\text{integrated}} = \delta^{15}\text{N}_{\text{initial}} + \varepsilon\{F\} \quad (4)$$

where  $F = \ln f \times f/(1 - f)$ . We refer to the N and O isotope effects derived from nitrate (and Eq. 3) as  $^{15}\varepsilon$  and  $^{18}\varepsilon$ , whereas we refer to the N isotope effect derived from particulate nitrogen (and Eq. 4) as  $^{15}\varepsilon_{\text{particulate}}$ .

Regression analyses of the linear models were computed with the statistical software Systat to ascertain the significance of the observed linear trends ( $p \leq 0.05$ ) and to obtain an estimate of the error associated with respective slopes ( $\varepsilon \pm$  standard error). Significant differences between (nitrate-derived)  $^{15}\varepsilon$  and  $^{18}\varepsilon$ , as well as between  $^{15}\varepsilon$  and  $^{15}\varepsilon_{\text{particulate}}$  within individual experiments were uncovered with a Student's  $t$ -test for slopes ( $p \leq 0.05$ ).

## Results

Derivation of the isotope effect,  $\varepsilon$ , based on the Rayleigh model fit of N and O isotopic measurements is illustrated in Fig. 1 for *T. pseudonana* grown in pFe19 medium. During exponential growth, nitrate was exponentially depleted from the medium (Fig. 1a), and the  $\delta^{15}\text{N}$  of the reactant pool (nitrate) and of the integrated product pool (particulate nitrogen) increased accordingly. Also shown in Fig. 1a is the concomitant increase in the  $\delta^{18}\text{O}$  of nitrate. The  $\delta^{15}\text{N}$  and  $\delta^{18}\text{O}$  of the reactant pool (i.e., nitrate) are plotted over the natural logarithm of the fraction of nitrate consumed

Table 1. Isotopic fractionation of nitrate N and O and particulate N during nitrate assimilation by marine phytoplankton grown in batch culture under various conditions.

Strain	Culture	pFe	Growth rate (d <sup>-1</sup> )	NO <sub>3</sub> <sup>-</sup> initial (μmol L <sup>-1</sup> )	NO <sub>3</sub> <sup>-</sup> final (μmol L <sup>-1</sup> )	<sup>15</sup> ε <sub>nitrate</sub> (±SE)	<sup>18</sup> ε <sub>nitrate</sub> (±SE)	n*	<sup>15</sup> ε <sub>particulate</sub> (±SE)
<i>E. huxleyi</i>	unstirred	19	1.1	154	83	19.7±0.5	19.7±0.5	6	n.d.†
<i>E. huxleyi</i>	unstirred	19	1.2	101	22	20.4±0.6	21.0±0.5	5	21.6±4.5‡
<i>T. oceanica</i>	unstirred	19	1.2	57	33	10.5±0.1	10.4±0.3	5	n.d.
<i>T. oceanica</i>	unstirred	21	0.9	51	4	8.9±0.5	7.8±0.3	5	9.0±1.2‡
<i>T. oceanica</i>	unstirred	21	0.7	156	103	13.4±1.8	12.6±0.9	7	20.6±3.1§
<i>T. pseudonana</i>	unstirred	19	1.4	146	63	6.7±0.2	5.8±0.8	7	9.9±1.8§§
<i>T. pseudonana</i>	unstirred	19	1.4	103	25	7.1±0.4	7.5±0.6	7	5.8±1.0§§
<i>T. weissflogii</i>	unstirred	19	1.4	70	44	13.0±0.2	14.3±0.2	4	n.d.
<i>T. weissflogii</i>	unstirred	19	1.4	68	39	12.2±0.4	10.8±0.7	4	n.d.
<i>T. weissflogii</i>	unstirred	20	0.9	76	56	9.1±0.4	10.3±1.5	4	n.d.
<i>T. weissflogii</i>	unstirred	20	1.0	75	41	10.4±0.4	11.1±0.5	4	n.d.
<i>T. weissflogii</i>	unstirred	20.5	0.9	50	13	9.2±0.2	10.0±0.4	5	10.5±4.3‡
<i>T. weissflogii</i>	unstirred	20.5	0.6	114	27	10.5±0.2	10.7±0.3	5	n.d.
<i>T. weissflogii</i>	unstirred	20.5	0.9	86	1	11.6±0.1	10.7±0.1	5	10.8±1.4
<i>T. weissflogii</i>	unstirred	20.5	0.9	83	4	11.4±0.4	11.5±0.6	4	12.8±1.5
<i>T. weissflogii</i>	stirred	19	1.7	84	25	12.8±0.4	14.2±0.4	9	n.d.
<i>T. weissflogii</i>	stirred	19	1.6	85	17	10.8±0.9	12.1±1.4	9	n.d.
<i>T. weissflogii</i>	stirred	20	1.4	74	31	13.2±0.7	13.1±0.6	8	n.d.
<i>T. weissflogii</i>	stirred	20	1.5	74	20	13.5±0.3	13.7±0.4	8	n.d.
<i>T. weissflogii</i>	stirred	21	0.7	80	65	9.2±0.8	6.1±1.0	9	n.d.
<i>T. weissflogii</i>	stirred	21	0.6	77	65	5.6±1.5	5.1±1.5	9	n.d.
<i>T. weissflogii</i>	uptake	19	—	72	15	14.9±0.2	14.2±0.1	6	n.d.
<i>T. weissflogii</i>	uptake	20	—	75	14	16.3±0.2	17.4±0.3	7	n.d.
<i>T. weissflogii</i>	uptake	20	—	78	16	16.9±0.3	18.2±0.3	7	n.d.

\* n refers to the sample number in the regressions for <sup>15</sup>ε as well as <sup>18</sup>ε.

† n.d., not determined.

‡ Slope (ε) of linear regression is not statistically significant.

§ Particulate <sup>15</sup>ε is significantly different from nitrate <sup>15</sup>ε.

|| Lowest [NO<sub>3</sub><sup>-</sup>] isotopic values were not considered in the regression analyses.

(ln *f*) in Fig. 1b. The slopes derived from the resultant linear relationship represent the respective N and O isotope effects, <sup>15</sup>ε and <sup>18</sup>ε (Eq. 3); it should be noted that they are not significantly different (Table 1). Figure 1b also illustrates the derivation of <sup>15</sup>ε from the slope of the linear relationship between the δ<sup>15</sup>N of the particulate nitrogen (i.e., the integrated product) and *F* (Eq. 4). In this experiment, the isotope effect computed from the reactant pool (<sup>15</sup>ε = 7.1 ± 0.4‰) and that from the integrated product (<sup>15</sup>ε<sub>particulate</sub> = 5.8 ± 1.0‰) differ significantly (Table 1), although this conclusion is cautionary since it is based on a relatively small sample size for the particulate measurements (i.e., *n* = 4; Table 1).

The kinetic isotope effects computed for all experiments are reported in Table 1. Unless flagged (‡), the isotope effects reported in Table 1 are the coefficients of statistically significant regression analyses (*p* ≤ 0.05). Strikingly apparent from the data in Table 1 is the large variation in nitrate-derived <sup>15</sup>ε, both among strains and within strains subjected to different experimental treatments. For *T. pseudonana*, <sup>15</sup>ε was in the lower portion of the observed range, around 7‰ to 8‰ for the two experiments conducted with this species. The highest values of <sup>15</sup>ε were observed for cultures of *E. huxleyi*, at 20‰ in two experiments. Values of <sup>15</sup>ε for *T. oceanica* ranged between 9‰ and 13‰ for

three experiments; a set of experiments intended to test for the impact of iron on the isotope effect showed no obvious trend.

The majority of the experiments reported in Table 1 were conducted with *T. weissflogii*. Nitrate-derived <sup>15</sup>ε ranged from 6‰ to 17‰. The lower values are associated with iron-limited cultures; however, not all iron-limited *T. weissflogii* cultures showed reduced <sup>15</sup>ε, such that the impact of low-iron conditions on the isotope effect (if any) remains unclear. The highest <sup>15</sup>ε measured for *T. weissflogii* (ca. 17‰) correspond to short-term nitrate uptake experiments at pFe20. *T. weissflogii* in stirred cultures showed no apparent differences in <sup>15</sup>ε across iron treatments compared to unstirred cultures, in spite of markedly higher growth rates in the stirred cultures at respective iron concentrations (Table 1).

The kinetic isotope effects derived from measurements of particulate N δ<sup>15</sup>N corresponded only modestly well to those derived from the δ<sup>15</sup>N of nitrate (Table 1). Significant differences were observed for the two experiments conducted with *T. pseudonana* (e.g., as in Fig. 1), but the sense of the differences was not internally consistent, since the nitrate-derived <sup>15</sup>ε was slightly higher than <sup>15</sup>ε<sub>particulate</sub> in one case but lower in the other. A large difference between nitrate-derived <sup>15</sup>ε and <sup>15</sup>ε<sub>particulate</sub> was observed in one experiment with *T.*

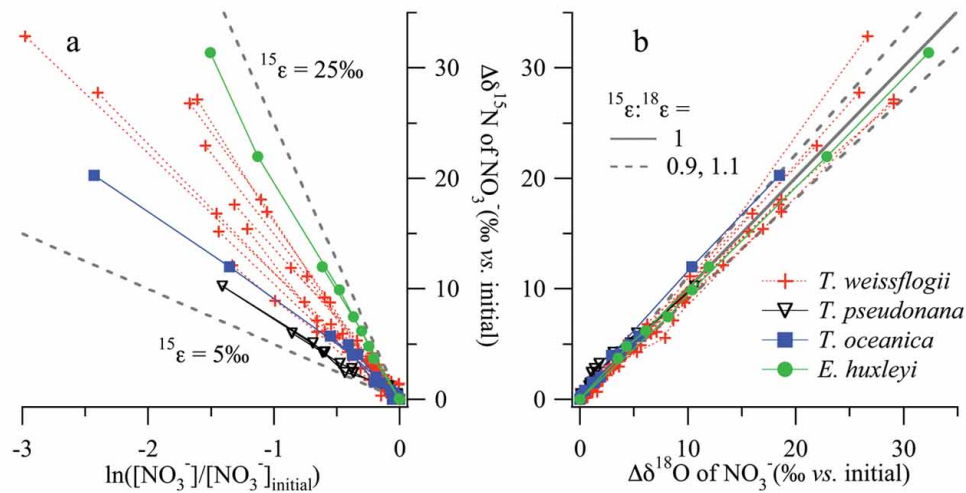


Fig. 2. Increase in nitrate  $\delta^{15}\text{N}$  and  $\delta^{18}\text{O}$  during nitrate assimilation by three species of diatoms and *E. huxleyi* grown under various environmental conditions (see Table 1). (a) Change in nitrate  $\delta^{15}\text{N}$  relative to initial nitrate  $\delta^{15}\text{N}$  plotted as a function of  $\ln f$ . The slopes of individual experiments define the corresponding N isotope effects on  $\text{NO}_3^-$  ( $^{15}\epsilon$ ) reported in Table 1. Dashed lines show slopes for  $^{15}\epsilon$  of 5‰ and 25‰. (b) The  $\delta^{15}\text{N}$  versus  $\delta^{18}\text{O}$  change in  $\text{NO}_3^-$ . The slope of the linear regression and its standard error ( $1.00 \pm 0.01$ ) are inclusive of all the experiments combined. The estimate of the standard error for the slope of the regression describes the error computed from the least-squares regression of sample means and does not take into account the measurement error associated with individual samples. Dashed lines show slopes of 1.1 and 0.9, for comparison.

*oceanica* grown in pFe21 medium, where  $^{15}\epsilon$  was 13.4%, while  $^{15}\epsilon_{\text{particulate}}$  was 20.6%. However, this discrepancy was not replicated in a similar experiment with *T. oceanica* (Table 1). We would not wish to emphasize the above discrepancies, since estimation of  $^{15}\epsilon_{\text{particulate}}$  was not a priority of this study, so our sampling was incomplete.

Like nitrogen, the oxygen atoms of nitrate underwent isotopic fractionation during nitrate assimilation, with an increase in  $\delta^{18}\text{O}$  during nitrate assimilation in all experiments (Figs. 1a, 2). Most striking is the observed coupling between the relative change in nitrate  $\delta^{15}\text{N}$  and that in  $\delta^{18}\text{O}$ . As illustrated in Fig. 2b, the linear regression of  $\delta^{15}\text{N}$  versus  $\delta^{18}\text{O}$  for the sum of the points in all of the experiments conducted indicates that the increase in nitrate  $\delta^{15}\text{N}$  throughout growth was matched by an equivalent increase in the  $\delta^{18}\text{O}$  of nitrate (slope =  $1.0 \pm 0.01$ ;  $p < 0.01$ ). Thus,  $^{15}\epsilon$  and  $^{18}\epsilon$  were similar regardless of algal species, the magnitude of the isotope effect, or culture conditions (Table 1). This  $^{15}\epsilon:^{18}\epsilon$  of  $\sim 1$  is indistinguishable from the  $^{15}\epsilon:^{18}\epsilon$  inferred from the nitrate  $\delta^{15}\text{N}$  and  $\delta^{18}\text{O}$  increase into the surface layer at station PAPA in the Gulf of Alaska (Casciotti et al. 2002).

The  $\delta^{15}\text{N}$  and  $\delta^{18}\text{O}$  were also coupled in the intracellular nitrate pool of *T. weissflogii* (Fig. 3). The  $\delta^{15}\text{N}$  of intracellular nitrate was elevated relative to medium nitrate (Fig. 3a, Needoba et al. in press). Taking the isotope data for intracellular and extracellular (medium) nitrate together, there is a strong correlation between the  $\delta^{15}\text{N}$  and  $\delta^{18}\text{O}$  of nitrate, with linear regression yielding a slope close to 1 (0.93; Fig. 3a). The isotopic ratios plotted in Fig. 3a are not normalized to the  $\delta^{15}\text{N}$  and  $\delta^{18}\text{O}$  of the initial nitrate stock, which can vary slightly among experiments. More importantly, part of the correlation in Fig. 3a is driven by the result described

above that medium nitrate tends to increase in  $\delta^{15}\text{N}$  and  $\delta^{18}\text{O}$  by a  $\sim 1:1$  ratio as consumption proceeds. To focus on the relationship between internal pool and medium nitrate, the difference between the  $\delta^{15}\text{N}$  and  $\delta^{18}\text{O}$  of internal nitrate versus external (medium) nitrate for each sampling of a culture is plotted in Fig. 3b, with linear regression again yielding a slope close to unity (0.95).

## Discussion

*The mechanism of isotope fractionation during nitrate assimilation*—As in higher plants, marine unicellular algae import nitrate with active transporters on the cell surface and reduce it to nitrite via an assimilatory nitrate reductase, an enzyme that resides in the cytoplasm (reviewed by Tischner 2000). The nitrite is subsequently reduced to ammonium by assimilatory nitrite reductase, which is located in the chloroplasts. The ammonium then undergoes incorporation into amino acids (Fig. 4).

Different investigators have proposed different causes for isotope fractionation during nitrate assimilation. Wada and Hattori (1978) first argued that N isotope fractionation by phytoplankton occurs during nitrate reduction, while Montoya and McCarthy (1995) ultimately favored fractionation associated with nitrate transport into the cell (Fig. 4). Understanding isotope fractionation during nitrate assimilation requires an understanding of nitrate assimilation itself, or at least aspects of it.

To maximize the activity of nitrate reductase, algal strains reportedly accumulate millimolar concentrations of nitrate in the cell (Dortch et al. 1984). Berges and Harrison (1995)

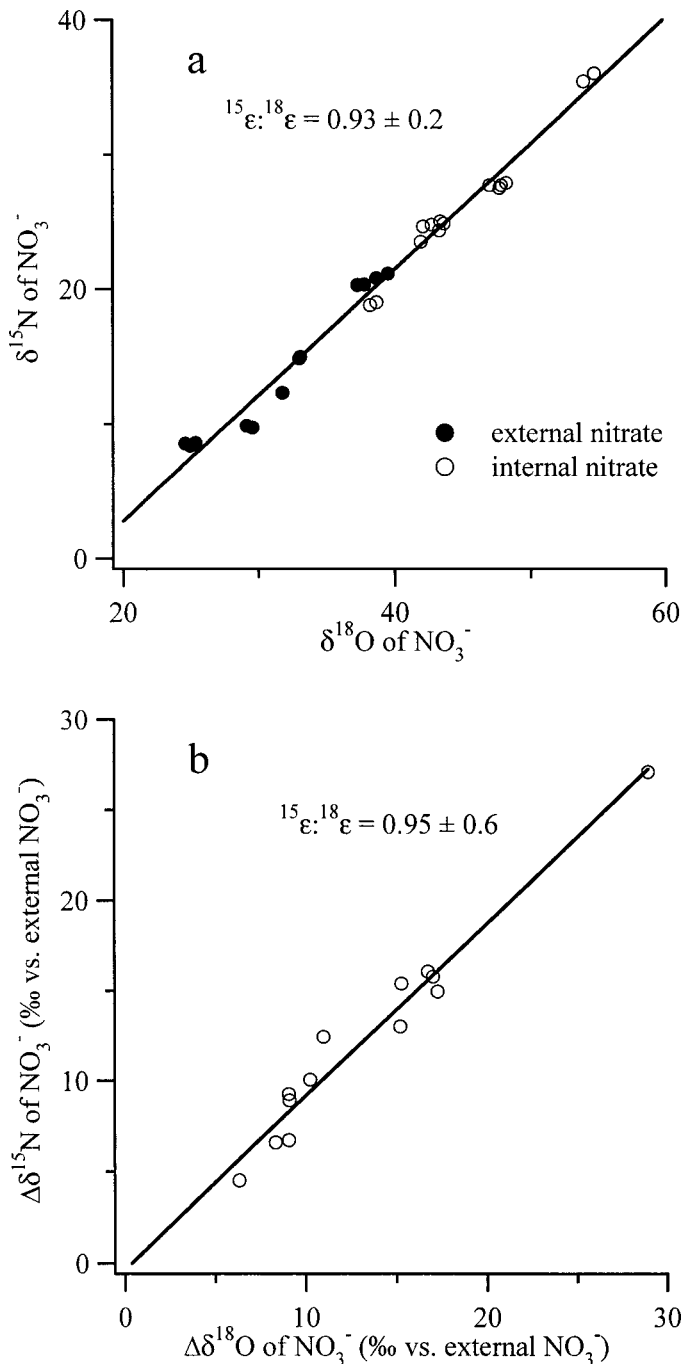


Fig. 3. Relationship between the N and O isotopic composition of nitrate in intracellular versus extracellular nitrate pools of *T. weissflogii* cultures grown under various environmental conditions (see Needoba et al. in press) for details of experimental treatments. (a) Nitrate  $\delta^{15}\text{N}$  versus  $\delta^{18}\text{O}$  for intracellular and extracellular nitrate. (b) Differences in nitrate  $\delta^{15}\text{N}$  and  $\delta^{18}\text{O}$  between intracellular and extracellular (medium) nitrate.

found that nitrate reductase activity of light-limited phytoplankton is highly correlated to net nitrate assimilation among different algal groups, which suggests that nitrate reduction is the rate-limiting step in nitrate assimilation, as seen in higher plants (Tischner 2000 and references therein).

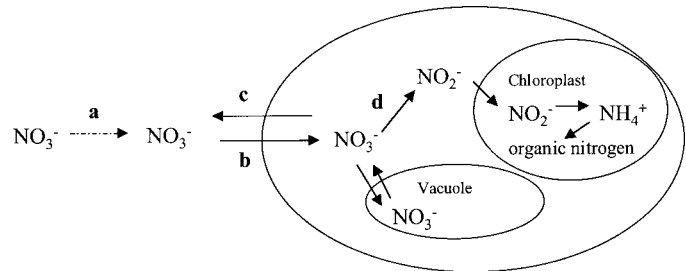


Fig. 4. Schematic depiction of nitrate uptake and assimilation by a phytoplankton cell. Hypothetically, isotope fractionation of nitrate could occur (a) at the diffusive boundary layer, (b) during uptake at the cell surface, and/or (d) during reduction to nitrite by nitrate reductase. (c) Represents potential nitrate efflux from the phytoplankton cell.

Nitrite, the product of nitrate reduction, generally does not accumulate within cells (Dortch et al. 1984), which indicates that the subsequent step of nitrite reduction is unlikely to limit assimilation. Thus, we expect that nitrate reduction represents the last possible step for the origin of isotope fractionation during nitrate assimilation.

The focus of Wada and Hattori (1978) on nitrate reduction as the driver of fractionation during assimilation was motivated in part by the expectation that transport cannot impart a significant isotope effect because it does not involve bond breakage. While chemical interactions must occur in the transporter, simple theory would suggest that this type of interaction is generally too weak to be important in stable isotope fractionation (Melander and Saunders 1980). Similarly, the slower diffusion of  $^{15}\text{N}$ - or  $^{18}\text{O}$ -bearing nitrate is also expected to be trivial ( $<1\%$ ) because of solvation and the molecular motion of water (O'Leary 1984; Hammond and Prokopenko in press). This view concurs qualitatively with work done on higher plants (Mariotti et al. 1982), cyanobacteria (Shearer et al. 1991), and diatoms (Wada and Hattori 1978). The lack of isotope fractionation during assimilation of nitrite by marine phytoplankton (Wada and Hattori 1978; Waser et al. 1998a) also supports the view that transport is a nonfractionating process. Since nitrite does not accumulate intracellularly, only uptake of nitrite at the cell surface has the potential to fractionate; yet no isotope effect is observed.

The enzyme nitrate reductase appears to impart a sizable N isotope fractionation, with several studies suggesting an isotope effect of 15–30‰ (Ledgard et al. 1985; Schmidt and Medina 1991), making nitrate reductase a reasonable candidate for driving the isotope effect associated with nitrate assimilation. However, this mechanism for fractionation requires a significant nitrate efflux from the cells in order to propagate the isotope effect extracellularly (Fig. 4). Given that energy must be spent to transport nitrate into the cell and that nitrate limits phytoplankton growth in large regions of the ocean, some investigators have doubted that the cell could be so open as to allow much expression of the nitrate reduction isotope effect. This has provided motivation for continued attention to transport as a possible source of nitrate isotope fractionation (Montoya and McCarthy 1995). However, nitrate efflux is routinely observed in higher

Table 2. Computed and empirical vibrational frequencies for the N-O bond of nitrate ( $^{15}\text{N}$ - and  $^{18}\text{O}$ -bearing), and corresponding calculated N and O isotope effects for dissociation of a single O atom from nitrate (hypothetical thermic decomposition). Also listed are measurements of the isotope effects for  $\text{Fe}^{2+}$  reduction of nitrate and reduction by nitrate reductases.

$\text{NO}_3^-$ isotopes	$\nu_i$ ( $\text{cm}^{-1}$ ) computed*	$\nu_i$ ( $\text{cm}^{-1}$ ) measured†	$\epsilon$ (‰)‡ computed $\nu_i$	$\epsilon$ (‰)‡ measured $\nu_i$	$\epsilon$ (‰) Fe(II)§	$\epsilon$ (‰) $\text{NO}_3^-$ reductase
$^{14}\text{N}^{16}\text{O}_3^-$	1,362	1,376				
$^{15}\text{N}^{16}\text{O}_3^-$	1,340	1,344	$^{15}\epsilon$ : 57	74	75	15–30
$^{14}\text{N}^{16}\text{O}_2^{18}\text{O}^-$	1,328	—	$^{18}\epsilon$ : 29			

\* From Hooke's law, with a vibrational force constant for the N-O bond of nitrate =  $8.2 \times 10^2 \text{ N m}^{-1}$  (see Brown and Drury 1967).

† By Begun and Fletcher (1960).

‡ Computed as in Brown and Drury (1967) with temperature = 298.15K.

§ Brown and Drury (1967).

|| Ledgard et al. (1985), Schmidt and Medina (1991).

plants, where it is believed to be dependent on internal nitrate concentrations (e.g., Ter Steege et al. 1999).

The isotopic fractionation mechanism for nitrate assimilation has remained equivocal in part because of the difficulty inherent in quantifying the isotopic composition of internal pools. Recently, Needoba et al. (in press) measured the concentration and  $\delta^{15}\text{N}$  of intracellular nitrate of *T. weissflogii* grown under various environmental conditions. As reported in previous studies, the authors found intracellular nitrate pools to be highly concentrated relative to the external media. More importantly, the intracellular nitrate was highly enriched in  $^{15}\text{N}$  relative to extracellular (medium) nitrate. As discussed by the authors, these results indicate that the isotope fractionation of nitrate reduction inside the cell is greater than that of nitrate uptake into the cell; if the rates of net uptake and reduction are equal (i.e., the internal nitrate pool is at steady state), then this implies that the isotope effect of reduction is greater than that of uptake. The authors then argued that observed N isotope effects were due in large part to N isotope fractionation imparted by nitrate reductase and were manifested extracellularly via nitrate efflux. Interestingly, intraspecies isotope effects were seemingly related to intracellular nitrate concentrations (Needoba et al. in press), suggesting that, as in higher plants, putative nitrate efflux may be dependent on intracellular nitrate concentrations. Moreover, the N isotope effects measured by Needoba et al. (in press) were also directly proportional to the difference in nitrate  $\delta^{15}\text{N}$  between internal and external pools, strongly incriminating nitrate efflux as the cause of variation in the isotope effect of nitrate uptake by *T. weissflogii*. In this conceptual model, the degree to which the isotope effect of nitrate reductase is expressed varies with the rate at which the  $^{15}\text{N}$ -enriched internal nitrate is effluxed from the cell relative to the rate at which it is reduced within the cell.

However, it has not yet been explicitly addressed whether the nitrate efflux from the cell is sufficiently high to explain the assimilation isotope effect as a result of nitrate reductase. Hypothetically, if nitrate efflux did not occur, the isotope effect imparted on nitrate by the reductase would not propagate out of the cell, and all of the intracellular nitrate would eventually be consumed. The  $^{15}\text{N}$  enrichment of external nitrate during nitrate assimilation would then be due solely to uptake at the cell surface. While the internal pool  $^{15}\text{N}$  data of Needoba et al. (in press) clearly show that the nitrate reductase isotope effect is greater than that of nitrate uptake

into the cell, they do not rule out the possibility of a significant isotope effect for nitrate uptake.

The coupled N and O isotope data presented here suggest an isotope effect driven solely by the reductase, and not by uptake at the cell surface. The  $^{15}\text{N}$  and  $^{18}\text{O}$  enrichment of external (medium) nitrate conform to a  $\sim 1:1$  trend for a broad range of N isotope effect (Fig. 2b). To the degree that different processes might impart different N:O ratios of isotopic fractionation, this suggests that a single mechanism is driving the  $^{15}\text{N}$  and  $^{18}\text{O}$  enrichment of external nitrate under all conditions and in all phytoplankton strains. Moreover, the N:O ratio of the heavy isotope enrichment in the intracellular nitrate relative to the medium is very close to 1:1 (Fig. 3), the same as observed in the temporal variations of the external nitrate. One could posit that fractionation is occurring at both the uptake and reduction steps, only that the isotope effect of nitrate reduction is greater (Needoba et al. in press). However, for both the intercellular-extracellular isotope difference and the progressive external nitrate isotope enrichment to occur with a  $^{15}\text{N}/^{14}\text{N}:^{18}\text{O}/^{16}\text{O}$  ratio of 1:1, both uptake and reduction of nitrate would need to have a  $^{15}\epsilon:^{18}\epsilon$  ratio of nearly 1:1.

We expect that  $^{15}\epsilon:^{18}\epsilon$  differs among various processes and even depends on their details. For instance, if molecular diffusion is inversely proportional to the square root of the molecular mass, then the  $^{15}\epsilon:^{18}\epsilon$  for nitrate diffusion would be  $\sim 0.5$ . A calculation based on transition-state theory of  $^{15}\epsilon:^{18}\epsilon$  for nitrate thermic N-O bond breakage (without any catalysis) yields  $^{15}\epsilon:^{18}\epsilon \sim 2$  (Table 2). In contrast, and quite remarkably, ab initio calculations using the NAP dissimilatory nitrate reductase from *Desulfovibrio desulfuricans* (Dias et al. 1999) as a model of the reaction center indicate a  $^{15}\epsilon:^{18}\epsilon$  of  $\sim 1$  (Zaharahiev pers. comm.). In any case, there is no reason to expect that several components of nitrate assimilation will all have a  $^{15}\epsilon:^{18}\epsilon$  of  $\sim 1$ . We do not know of measurements that provide insight on the expected  $^{15}\epsilon:^{18}\epsilon$  for binding and release of nitrate in a transporter, but our prediction is that the  $^{15}\epsilon:^{18}\epsilon$  will be low for this process because a modest intermolecular interaction such as this is more likely to depend on the isotope of the O atoms, which are more readily available to interact with the transporter. In general, the observation of  $^{15}\epsilon:^{18}\epsilon \sim 1$  for a wide range in  $^{15}\epsilon$  suggests that only one process drives the fractionation for this entire range. This fits well with the model posed above, where the fractionation of nitrate N and O isotopes

is driven entirely by nitrate reduction and the amplitude of the assimilation isotope effect is modulated by the degree to which intracellular nitrate is able to efflux from the cell before being reduced. The  $^{15}\epsilon:^{18}\epsilon$  of  $\sim 1$  that applies at  $^{15}\epsilon$  of 20‰ also applies at  $^{15}\epsilon$  of 10‰ and apparently even lower, arguing for the same fractionation mechanism across this range, such that transport is not a major contributor to fractionation even when the latter is slight.

It is worth noting that nitrate reductase is also found embedded in the plasma membrane of plant and algal cells (Tischner 2000 and references therein). Plasmalemma nitrate reductase has been hypothesized to participate in nitrate uptake, coupling transport across the membrane with nitrate reduction. In such a case, reduction could drive the isotope fractionation without requiring the efflux of nitrate from the cell. However, the large accumulations of nitrate in the intracellular pool and the isotopic enrichment of nitrate suggest that a sizable amount of the nitrate reduction is occurring within the cytoplasm, arguing against a dominant role for this external nitrate reductase in the isotope effect of nitrate assimilation. Moreover, the putative role of plasmalemma-bound nitrate reductase in nitrate transport has been challenged (e.g., Mora et al. 2002) and remains ambiguous.

*The magnitude of the N isotope effect during nitrate assimilation*—We have argued above that nitrate reductase comprises the chief fractionating step in nitrate assimilation, with its expression occurring by the efflux of intracellular nitrate back into the environment. This suggests that the amplitude and variability of the isotope effect will largely be determined by the rate of this efflux relative to the rate of nitrate reduction (the efflux/reduction ratio to which we refer below). One might infer from the low amplitude of  $^{15}\epsilon$  in the open ocean relative to cultures (and its apparently lower degree of variability) that nitrate efflux tends to be constant and minimal in the open ocean.

However, we do not understand the controls on the nitrate efflux/reduction ratio and, thus, cannot predict  $^{15}\epsilon$ , even in our controlled cultures. Apparent from our observations and those of other studies (Wada and Hattori 1978; Montoya and McCarthy 1995; Waser et al. 1998b; Needoba et al. 2003) is a seeming lack of species or algal group specificity relating to  $^{15}\epsilon$ . While some  $^{15}\epsilon$  values obtained in this study for *T. pseudonana* and *T. weissflogii* agree with some previous reports (Montoya and McCarthy 1995; Waser et al. 1998a), the isotope effect of 20‰ observed here for *E. huxleyi* is excessively high compared to previous estimates of 4‰ (Waser et al. 1998a; Needoba et al. 2003), although culture conditions were roughly similar. This study presents the first published values of  $\epsilon$  for *T. oceanica*. These fell in the range observed here for *T. weissflogii*.

While large intraspecific variations in  $^{15}\epsilon$  point strongly to an effect of growth conditions, our study does not provide a clear picture of this effect. Our attempt to define a role for iron limitation in modulating  $^{15}\epsilon$  yielded no conclusive trend. Although iron limitation appeared to cause a decrease in  $^{15}\epsilon$  in some experiments with *T. weissflogii*, not all experiments concurred (with *T. weissflogii* or with *T. oceanica*). Similarly, Needoba et al. (in press) reported no significant changes in isotope effect of *T. weissflogii* grown in iron-deplete me-

dium. The lower  $^{15}\epsilon$  observed in some of the low-iron experiments may be due to a decrease in nitrate uptake rates and lower intracellular nitrate concentrations due to iron stress, resulting in lower nitrate efflux.

Shaking of the cultures caused a marked increase in the growth rates of *T. weissflogii* compared to analogous treatments in unstirred cultures, but no concomitant changes in isotope effects were observed. Although net nitrate uptake was significantly higher in the stirred cultures, the lack of a clear difference in  $^{15}\epsilon$  implies that the efflux/reduction ratio was similar in shaken and unstirred cultures.

We observed a higher  $^{15}\epsilon$  for nitrate assimilation by *T. weissflogii* cells that were resuspended in fresh medium compared to cell cultures initiated from small inocula. We did not expect this result, reasoning that exponential phase cells would be at a physiological steady state with respect to nitrate assimilation, such that resuspension into fresh medium would cause no change in the isotope effect. It is possible that the cells were experiencing N limitation in late exponential growth prior to resuspension, or that filtration and resuspension affected the integrity or permeability of the cell membrane. Either of these factors might have increased the nitrate efflux/reduction ratio: previous N limitation could result in overexpression of nitrate uptake and high intracellular nitrate concentrations following resuspension in nitrate-bearing medium, and hence a higher efflux/reduction ratio, while filtration could also contribute to higher efflux if membrane permeability were affected.

Popp et al. (1998) found that cell geometry, namely, cell surface area to volume ratio, is one of a few major controls on carbon isotopic fractionation during inorganic carbon assimilation. By analogy, a higher surface area to volume ratio could potentially lead to higher cellular efflux of nitrate for a given rate of nitrate reduction. No systematic relationship between surface area to volume ratio and N isotopic fractionation was apparent in our study, whether among the algal species in our experiments or for a set of experiments run with a single species (data not shown). Our cell surface area and volume estimates were inferred from Coulter counter measurements of cell diameter and with consideration of cell shape in only a rudimentary way, so we cannot rule out that future studies will reveal such a relationship. Generally, though, we expect that the isotope effect of nitrate assimilation is the result of multiple additional factors (e.g., vacuolar space, nitrate transporter density, and nitrate reductase concentration) that are regulated by the cells on the basis of resource availability and overall cellular energetics (Needoba and Harrison in press). As with the isotope dynamics of carbon assimilation, much work is required to develop a fully mechanistic understanding.

The culture experiments described in this study indicate that phytoplankton fractionate the N and O isotopes of nitrate with a  $^{15}\epsilon:^{18}\epsilon$  ratio of  $\sim 1$ , regardless of the absolute isotope effect. A similar 1:1 increase in  $\delta^{15}\text{N}$  and  $\delta^{18}\text{O}$  has been observed in surface ocean nitrate that has undergone nitrate assimilation by resident plankton (Casciotti et al. 2002). Isotopic analyses of intracellular nitrate indicate a  $\sim 1:1$  N:O isotope enrichment of this nitrate relative to external nitrate. The internal pool observations suggest that

assimilatory nitrate reductase has an intrinsic  $^{15}\epsilon:^{18}\epsilon$  of around 1. Moreover, the consistency of the internal pool  $^{15}\epsilon:^{18}\epsilon$  with that of external nitrate suggests that a single fractionating mechanism is at work in all studied phytoplankton strains and under all studied growth conditions, regardless of the amplitude of  $^{15}\epsilon$  that is expressed. If there were more than two significant fractionating mechanisms, then both would need to have a  $^{15}\epsilon:^{18}\epsilon$  of 1, and theoretical calculations of  $^{15}\epsilon:^{18}\epsilon$  for various processes indicate that this is highly unlikely (Table 2). Together, these data strongly support the scenario that nitrate reductase is the dominant source of isotope fractionation in nitrate assimilation by phytoplankton, even at low  $^{15}\epsilon$  ( $\sim 6\text{‰}$ ). If this is correct, then the range of isotope effects observed for nitrate assimilation by phytoplankton cultures is driven by variations in the degree to which nitrate efflux across the cell boundary enables full expression of the large isotope effect intrinsic to nitrate reductase ( $\sim 15\text{--}30\text{‰}$ ; Ledgard et al. 1985; Schmidt and Medina 1991). It is noteworthy that this model is analogous to that of isotope fractionation for photosynthetic carbon dioxide fixation by  $C_3$  plants (O'Leary 1981).

Another process likely to affect the N and O isotopic signatures of nitrate in the ocean is denitrification. Although N and O isotopic coupling has not been investigated for laboratory cultures of denitrifying bacteria, nitrate N and O isotopic measurements in denitrifying zones suggest that the  $^{15}\epsilon:^{18}\epsilon$  of denitrification is also indistinguishable from 1 (Sigman et al. 2003). One might infer that, as with nitrate assimilation, the isotope effect observed in external nitrate is due to the isotope effect intrinsic to nitrate reduction, with transport of the  $^{15}\text{N}$ - and  $^{18}\text{O}$ -enriched intracellular nitrate back into the environment. Water column data from various regions yield a remarkably consistent estimate of 25–30‰ for the isotope effect for denitrification (e.g., Cline and Kaplan 1975; Liu and Kaplan 1989; Brandes et al. 1998; Sigman et al. 2003). This value is close to the maximum value from denitrifier cultures, 25–30‰ (e.g., Mariotti et al. 1981). One possible explanation for this similarity is that denitrifiers universally allow rapid efflux of nitrate into the environment, so that the isotope effect intrinsic to nitrate reductase is always fully expressed. Such "open system" behavior for intracellular nitrate is sensible for marine denitrifiers (as opposed to phytoplankton) in that ambient  $[\text{NO}_3^-]$  is typically quite high in zones of water column denitrification, weakening the competitive advantage associated with nitrate storage.

Although multiple forms of nitrate reductase catalyze the reduction of nitrate to nitrite, and assimilatory and dissimilatory forms differ, all share a molybdenum reaction center and likely yield similar transition-state structures of Mo-bound nitrate at the active site. It follows that the N and O isotope effects of all nitrate reductases, whether assimilatory or dissimilatory, seem likely to show similar fractionation characteristics (see Melander and Saunders 1980). In this sense, the concordance of the available data with a  $^{15}\epsilon:^{18}\epsilon$  of  $\sim 1$  for both nitrate assimilation and denitrification is consistent with the view that nitrate reductase is the driver of fractionation in both processes.

However, in contrast with oceanic results, measurements of N and O isotopes of nitrate in freshwater systems suggest

a  $^{15}\epsilon:^{18}\epsilon$  of 1.4 to 2 for denitrification (Lehmann et al. 2003). This discrepancy is remarkable and difficult to explain, given the data in hand. We are presently working to characterize the  $^{15}\epsilon:^{18}\epsilon$  of denitrification in bacteria isolated from marine and terrestrial environments.

The use of coupled N and O isotopic measurements of nitrate represents a potentially powerful tool for studying the oceanic N cycle. Nitrate  $^{15}\text{N}/^{14}\text{N}$  shares a limitation with other geochemical tools used to the study N cycle (e.g., nitrate concentration and nitrate-to-phosphate ratio) that it records regional imbalances in the counteracting fluxes of the N cycle but does not illuminate the gross rates of these fluxes. Notably, in the case of nitrate assimilation, neither the N isotopes nor the other available tracers are able to separate nitrate assimilation from the organic matter remineralization and nitrification that recycles the assimilated N back into nitrate. Similarly, the N isotope signals of nitrogen fixation and denitrification interfere destructively, removing both signals. Separating the impacts of these processes on the nitrate concentration of seawater, both at the surface and in the ocean interior, would greatly advance our understanding of ocean biogeochemistry. The use of the coupled N and O isotopes of nitrate allows distinction between the processes that otherwise overprint one another (Sigman et al. 2003; Lehmann et al. in press), thereby providing a new and important tool in study of the N cycle. Toward this end, the data reported here provide a fundamental constraint on the behavior of N and O isotopes of nitrate.

## References

- ALTABET, M. A. 2001. Nitrogen isotopic evidence for micronutrient control of fractional  $\text{NO}_3^-$  utilization in the equatorial Pacific. *Limnol. Oceanogr.* **46**: 368–380.
- , AND R. FRANÇOIS. 1994. Sedimentary nitrogen isotopic ratio as a recorder for surface ocean nitrate utilization. *Glob. Biogeochem. Cycles* **8**: 103–116.
- BEGUN, G. M., AND W. H. FLETCHER. 1960. Vibrational spectra of isotopic nitric acids. *J. Chem. Phys.* **33**: 1083.
- BORGES, J. A., AND P. J. HARRISON. 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 three species of marine phytoplankton. *Limnol. Oceanogr.* **40**: 82–93.
- BÖHLKE, J. K., S. J. MROCKOWSKI, AND T. B. COPLEN. 2003. Oxygen isotopes in nitrate: new reference materials for  $^{18}\text{O}:^{17}\text{O}:^{16}\text{O}$  measurements and observations on nitrate-water equilibration. *Rapid Commun. Mass Spectrom.* **17**: 1835–1846.
- BRAMAN, R. S., AND S. A. HENDRIX. 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., A. H. DEVOL, T. YOSHINARI, D. A. JAYAKUMAR, AND S. W. A. NAQVI. 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.
- BROWN, L. L., AND J. S. DRURY. 1967. Nitrogen-isotope effects in the reduction of nitrate, nitrite, and hydroxylamine to ammonia. I. In sodium hydroxide solution with Fe(II). *J. Chem. Phys.* **46**: 2833–2837.
- CASCIOTTI, K. L., D. M. SIGMAN, M. G. HASTINGS, J. K. BOHLKE, AND A. HILKERT. 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 I. R. KAPLAN. 1975. Isotopic fractionation of dissolved nitrate during denitrification in the Eastern Tropical North Pacific Ocean. *Mar. Chem.* **3**: 271–299.
- DIAS, J. M., AND OTHERS. 1999. Crystal structure of the first dissimilatory nitrate reductase at 1.9 angstrom solved by MAD methods. *Struct. Fold. Des.* **7**: 65–79.
- DORTCH, Q., J. R. CLAYTON, S. S. THORENSEN, AND S. I. AHMED. 1984. Species differences in accumulation of nitrogen pools in phytoplankton. *Mar. Biol.* **81**: 237–250.
- FARRELL, J. W., T. F. PEDERSEN, S. E. CALVERT, AND B. NIELSEN. 1995. Glacial-interglacial changes in nutrient utilization in the equatorial Pacific Ocean. *Nature* **377**: 514–517.
- FRANÇOIS, R., M. A. ALTABET, AND L. H. BURKLE. 1992. Glacial to interglacial changes in surface nitrate utilization in the Indian sector of the Southern Ocean as recorded by sediment  $\delta^{15}\text{N}$ . *Paleoceanography* **7**: 589–606.
- , AND OTHERS. 1997. Water column stratification in the Southern Ocean contributed to the lowering of glacial atmospheric  $\text{CO}_2$ . *Nature* **389**: 929–935.
- GONFIANTINI, R., W. STICHLER, AND K. ROZANSKI. 1995. Standards and intercomparison materials distributed by the International Atomic Energy Agency for stable isotope measurements, pp. 13–29. *In* Reference and Intercomparison Material of Stable Isotopes of Light Elements. IAEA-TECDOC-825, Vienna.
- HAMMOND, D. E., AND M. PROKOPENKO. In press. Differential diffusivity of light stable isotopes in water and seawater:  $^{15}\text{N}/^{14}\text{N}$  in ammonium. *Geochim. Cosmochim. Acta*.
- KELLER, M. D., W. K. BELLOWES, AND R. R. L. GUILLARD. 1988. Microwave treatment for sterilization of phytoplankton culture media. *J. Exp. Mar. Biol. Ecol.* **117**: 279–283.
- LEDGARD, S. F., K. C. WOO, AND F. G. BERGERSEN. 1985. Isotopic fractionation during reduction of nitrate to nitrite by extracts of spinach leaves. *Aust. J. Plant Phys.* **12**: 631–640.
- LEHMANN, M. F., P. REICHERT, S. M. BERNASCONI, A. BARBIERI, AND J. A. MCKENZIE. 2003. Modeling nitrogen and oxygen isotope fractionation during denitrification in a lacustrine redox-transition zone. *Geochim. Cosmochim. Acta.* **67**: 2529–2542.
- , D. M. SIGMAN, AND W. M. BERELSON. 2004. Coupling the  $^{15}\text{N}/^{14}\text{N}$  and  $^{18}\text{O}/^{16}\text{O}$  of nitrate as a constraint on benthic nitrogen cycling. *Mar. Chem.* **88**: 1–20.
- LIU, K. -K., AND I. R. KAPLAN. 1989. The eastern tropical Pacific as a source of  $^{15}\text{N}$ -enriched nitrate in seawater off southern California. *Limnol. Oceanogr.* **34**: 820–830.
- MARIOTTI, A., J. C. GERMON, P. HUBERT, P. KAISER, R. LETOLLE, A. TARDIEUX, AND P. TARDIEUX. 1981. Experimental determination of nitrogen kinetic isotope fractionation: Some principles; illustration for the denitrification and nitrification processes. *Plant Soil* **62**: 413–430.
- , F. MARIOTTI, M. L. CHAMPIGNY, N. AMARGER, AND A. MOYSE. 1982. Nitrogen isotope fractionation associated with nitrate reductase activity and uptake of  $\text{NO}_3^-$  by Pearl-Millet. *Plant Physiol.* **69**: 880–884.
- MELANDER, L., AND W. H. J. SAUNDERS. 1980. Reaction rates of isotopic molecules. Wiley.
- MONTOYA, J. P., AND J. J. MCCARTHY. 1995. Isotopic fractionation during nitrate uptake by marine phytoplankton grown in continuous culture. *J. Plankton Res.* **17**: 439–464.
- MORA, C., F. G. WITT, P. J. APARICIO, M. QUIÑONES. 2002. Independent induction of two blue light-dependent monovalent anion transport systems in the plasma membrane of *Monoraphidium braunii*. *J. Exp. Bot.* **53**: 1909–1918.
- NEEDOBA, J. A., AND P. J. HARRISON. In press. Influence of low light and light/dark cycle on  $\text{NO}_3^-$  uptake, intracellular  $\text{NO}_3^-$  and nitrogen isotope fractionation by marine phytoplankton. *J. Phycol.*
- , D. M. SIGMAN, P. J. HARRISON. In press. The mechanism of isotope fractionation by algal nitrate assimilation as illuminated by the  $^{15}\text{N}/^{14}\text{N}$  of intracellular nitrate. *J. Phycol.*
- , N. A. WASER, P. J. HARRISON, AND S. E. CALVERT. 2003. Nitrogen isotope fractionation in 12 species of marine phytoplankton during growth on nitrate. *Mar. Ecol. Prog. Ser.* **255**: 81–91.
- O'LEARY, M. H. 1981. Carbon isotope fractionation in plants. *Phytochemistry* **20**: 553–567.
- . 1984. Measurement of the isotope fractionation associated with diffusion of carbon dioxide in aqueous solution. *J. Phys. Chem.* **88**: 823–825.
- POPP, B. N., E. A. LAWS, R. R. BIDIGARE, J. E. DORE, K. L. HANSON, AND S. G. WAKEHAM. 1998. Effect of phytoplankton cell geometry on carbon isotopic fractionation. *Geochim. Cosmochim. Acta* **62**: 69–77.
- PRICE, N. M., G. I. HARRISON, J. G. HERRING, R. J. HUDSON, P. M. V. NIREL, B. PALENIK, AND F. M. M. MOREL. 1988/1989. Preparation and chemistry of the artificial algal culture medium Aquil. *Biol. Oceanogr.* **6**: 443–461.
- REVESZ, K., J. K. BÖHLKE, AND T. YOSHINARI. 1997. Determination of  $\delta^{18}\text{O}$  and  $\delta^{15}\text{N}$  in nitrate. *Anal. Chem.* **69**: 4375–4380.
- SCHMIDT, H. L., AND R. MEDINA. 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., J. D. SCHNEIDER, AND D. H. KOHL. 1991. Separating the efflux and influx components of net nitrate uptake by *Synechococcus*-R2 under steady-state conditions. *J. Gen. Microbiol.* **137**: 1179–1184.
- SIGMAN, D. M., M. A. ALTABET, R. FRANÇOIS, AND G. FISCHER. 1999a. The  $\delta^{15}\text{N}$  of nitrate in the Southern Ocean: Consumption of nitrate in surface waters. *Glob. Biogeochem. Cycles* **13**: 1149–1166.
- , ———, ———, D. C. MCCORKLE, AND J.-F. GAILLARD. 1999b. The isotopic composition of diatom-bound nitrogen in Southern Ocean sediments. *Paleoceanography* **14**: 118–134.
- , ———, R. H. MICHENER, D. C. MCCORKLE, B. FRY, AND R. M. HOLMES. 1997. Natural abundance-level measurement of the nitrogen isotopic composition of oceanic nitrate: An adaptation of the ammonia diffusion method. *Mar. Chem.* **57**: 227–242.
- , K. L. CASCIOTTI, M. ANDREANI, C. BARFORD, M. GALANTER, AND J. K. BOHLKE. 2001. A bacterial method for the nitrogen isotopic analysis of nitrate in seawater and freshwater. *Anal. Chem.* **73**: 4145–4153.
- , R. ROBINSON, A. N. KNAPP, A. VAN GEEN, D. C. MCCORKLE, J. A. BRANDES, AND R. C. THUNELL. 2003. Distinguishing between water column and sedimentary denitrification in the Santa Barbara Basin using the stable isotopes of nitrate. *Geochem. Geophys. Geosyst.* **4**: 1040–1059.
- SILVA, S. R., C. KENDALL, D. H. WILKINSON, A. C. ZIEGLER, C. C. Y. CHANG, AND R. J. AVANZINO. 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.
- TER STEEGE, M. W., I. STULEN, P. K. WIERSEMA, F. POSTHUMUS, AND W. VAALBURG. 1999. Efficiency of nitrate uptake in spinach: Impact of external nitrate concentration and relative growth rate on nitrate influx and efflux. *Plant Soil* **208**: 125–134.
- THORENSEN, S. S., Q. DORTCH, AND S. I. AHMED. 1982. Comparison of methods for extracting intracellular pools of inorganic nitrogen from marine phytoplankton. *J. Plankton Res.* **4**: 695–704.
- TISCHNER, R. 2000. Nitrate uptake and reduction in higher and lower plants. *Plant Cell Environ.* **23**: 1005–1024.

- WADA, E. 1980. Nitrogen isotope fractionation and its significance in biogeochemical processes occurring in marine environments, pp. 375–398. *In* E. Goldberg, Y. Horibe, and K. Saruhashi [eds.], *Isotope marine chemistry*. Uchida Rokakuho.
- , AND A. HATTORI. 1978. Nitrogen isotope effects in the assimilation of inorganic nitrogenous compounds. *Geomicrobiol. J.* **1**: 85–101.
- WASER, N. A., D. H. TURPIN, P. J. HARRISON, B. NIELSEN, AND S. E. CALVERT. 1998*a*. Nitrogen isotope fractionation during the uptake and assimilation of nitrate, nitrite, and urea by a marine diatom. *Limnol Oceanogr.* **43**: 215–224.
- , K. D. YIN, Z. M. YU, K. TADA, P. J. HARRISON, D. H. TURPIN, AND S. E. CALVERT. 1998*b*. 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.
- WESTALL, J. C., J. L. ZACHARY, AND F. M. M. MOREL. 1976. MINEQL: A computer program for the calculation of chemical equilibrium composition in aqueous systems. Tech. Note 18. MIT Univ. Press.
- WU, J., S. E. CALVERT, AND C. S. WONG. 1997. Nitrogen isotope variations in the subarctic Pacific northeast Pacific: Relationships to nitrate utilization and trophic structure. *Deep-Sea Res. I* **44**: 287–314.

*Received: 24 September 2003*

*Accepted: 30 March 2004*

*Amended: 16 April 2004*