

# Nitrogen and oxygen isotope fractionation during dissimilatory nitrate reduction by denitrifying bacteria

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## Abstract

We report the first measurements of coupled nitrogen (N) and oxygen (O) isotope fractionation of nitrate by laboratory cultures of denitrifying bacteria. Two seawater strains (*Pseudomonas stutzeri*, *Ochrobactrum* sp.) and three freshwater strains (*Paracoccus denitrificans*, *Pseudomonas chlororaphis*, *Rhodobacter sphaeroides*) were examined. Among four strains of facultative anaerobic denitrifiers, N and O isotope effects were variable, ranging from 5‰ to 25‰, with evidence for a drop in the isotope effects as nitrate concentrations approached the half-saturation constant for nitrate transport. O isotope effects were similar to their corresponding N isotope effect, such that the progressive increase in nitrate  $\delta^{18}\text{O}$ , when plotted against that in  $\delta^{15}\text{N}$  (where  $\delta^{18}\text{O}_{\text{sample}} = [({}^{18}\text{O}:{}^{16}\text{O})_{\text{sample}}/({}^{18}\text{O}:{}^{16}\text{O})_{\text{reference}} - 1] \times 1000$ , and  $\delta^{15}\text{N}_{\text{sample}} = [({}^{15}\text{N}:{}^{14}\text{N})_{\text{sample}}/({}^{15}\text{N}:{}^{14}\text{N})_{\text{reference}} - 1] \times 1000$ ), yielded slopes of 0.86 to 1.02, with a mean value of 0.96. *R. sphaeroides*, a photo-heterotroph that possesses only a periplasmic (nonrespiring) dissimilatory nitrate reductase, showed less variability in nitrate N isotope effects, between 13‰ and 20‰, with a modal value of ~15‰. In contrast to the respiratory denitrifiers, *R. sphaeroides* consistently showed a distinct ratio of  $\delta^{18}\text{O}$  to  $\delta^{15}\text{N}$  change of ~0.62. We hypothesize that heavy N and O isotope discrimination during respiratory denitrification occurs during the intracellular reduction of nitrate by the respiratory nitrate reductase, and the observed magnitude of fractionation is likely regulated by the ratio of cellular nitrate efflux relative to uptake. The data for *R. sphaeroides* are consistent with isotope discrimination directly reflecting the N and O isotope effects of the periplasmic nitrate reductase NAP, without modification by nitrate uptake and efflux.

Denitrification is the principal mechanism by which fixed nitrogen in the ocean is lost to the atmosphere. It involves the bacterially mediated reduction of nitrate ( $\text{NO}_3^-$ ) and nitrite ( $\text{NO}_2^-$ ) to gaseous nitrous oxide ( $\text{N}_2\text{O}$ ) and dinitrogen ( $\text{N}_2$ ). The occurrence of denitrification in the environment is often evident from its nitrogen isotopic imprint on ambient nitrate. The dissimilatory reduction of nitrate to nitrite exhibits significant nitrogen (N) isotope discrimination, where  $^{14}\text{N}$ -bearing nitrate is consumed more rapidly than that bearing  $^{15}\text{N}$ , leaving the remaining substrate pool with an elevated  $^{15}\text{N}:^{14}\text{N}$  ratio. The extent

against which heavy isotopes are discriminated in a unidirectional biological reaction such as denitrification can be quantified in terms of the kinetic isotope effect,  $\epsilon$ . This parameter is a measure of the ratio of the reaction rate coefficients for the light vs. heavy isotope:  $\epsilon = ([k_{\text{light}}/k_{\text{heavy}}] - 1) \times 1000$ , when expressed in per mil (‰). Culture studies of denitrifying bacterial isolates have yielded a broad range of nitrate N isotope effects ( $^{15}\epsilon$ ) for various denitrifiers, from 2‰ to 30‰ (Table 1, and citations therein). A similarly broad range of N isotope effects is reported for denitrification in freshwater and terrestrial environments, whereas isotope effects estimated for open-ocean systems are consistently high, ranging between 20‰ and 30‰.

With new methods for nitrate isotope analysis in seawater samples (Sigman et al. 2001; Casciotti et al. 2002; McIlvin and Altabet 2005), investigation of the coupled fractionation of the N and O ( $^{18}\text{O}:^{16}\text{O}$ ) isotopes of nitrate in the ocean are beginning (Lehmann et al. 2004; Sigman et al. 2005; Wankel et al. 2007). The first published ocean depth profile of coupled nitrate N and O isotope ratios from the subarctic Pacific revealed a 1:1 covariation of the N and O isotope enrichments associated with nitrate assimilation in the euphotic zone (Casciotti et al. 2002). Subsequent lab studies demonstrated that phytoplankton

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## Acknowledgments

We dedicate this work to the memory of Edward Steifel. We thank B. Song for providing bacterial strains, F. M. M. Morel and T. Spiro for input during the experimental stage of this work, M. Rohde for assistance in making culture media, and G. Cane and Y. Wang for isotopic analyses.

This work was supported by a seed grant from Center for Bioinorganic Chemistry (CEBIC, funded by the United States National Science Foundation; F. M. M. Morel, Director), a postgraduate grant from Canada's National Science and Engineering Research Council to J.G., and grant OCE-0447570 from the United States National Science Foundation to D.M.S.

Table 1. Summary of existing values measured for nitrogen (N) isotope effects imparted on nitrate by denitrification in laboratory cultures and in environmental samples.

Experimental system	Conditions	$^{15}\epsilon$ (‰)	Reference
<i>Pseudomonas stutzeri</i>	Batch culture	20–30	Wellman et al. 1968
<i>Paracoccus denitrificans</i>	Batch culture	13–20	Delwiche and Steyn 1970
<i>Paracoccus denitrificans</i>	Chemostats	28.6	Barford et al. 1999
Marine denitrifier		14–21	Miyake and Wada 1971
Freshwater denitrifier		2–12	Wada et al. 1975
Soil	Glucose added	14–23	Blackmer and Bremner 1977
Soil	20°C	29.4±2.4	Mariotti et al. 1981
Soil	30°C	24.6±0.9	Mariotti et al. 1981
Groundwater	Kalahari	30±6	Vogel et al. 1981
Groundwater	Gravelly sands	15.9	Böttcher et al. 1990
Groundwater	Septic sands	22.9	Aravena and Robertson 1998
Lake Lugano		20.7	Lehmann et al. 2003
Eastern Tropical North Pacific		20–30	Brandes et al. 1998
Eastern Tropical North Pacific		30	Voss et al. 2001
Central Arabian Sea		22–25	Brandes et al. 1998

cultures indeed fractionate the N and O isotopes of nitrate with equivalent isotope effects during nitrate assimilation (Granger et al. 2004). Measurements of the isotopic composition of nitrate in freshwater and in marine systems suggest that denitrification also imparts proportional heavy atom enrichment on the N and O atoms of nitrate (Lehmann et al. 2003; Sigman et al. 2005). Nitrate isotope data from suboxic oxygen minimum zones in the ocean show increases in nitrate  $\delta^{18}\text{O}$  and  $\delta^{15}\text{N}$  associated with denitrification that follow a trajectory of 1:1 or greater, with deviations above unity attributed to additional N-transforming processes (Sigman et al. 2005). In contrast to ocean environments, nitrate N and O isotope fractionation observed for denitrification in groundwater and in lakes is associated with a  $\delta^{18}\text{O}$  to  $\delta^{15}\text{N}$  fractionation ratio (henceforth referred to as  $\Delta\delta^{18}\text{O}:\Delta\delta^{15}\text{N}$ ) of  $\sim 0.6$  (Lehmann et al. 2003, and references therein). This striking difference between freshwater and marine systems is unexplained.

The coupled nitrate N and O isotope discrimination during denitrification has not been verified in culture, and the kinetic O isotope effect of denitrification ( $^{18}\epsilon$ ) is poorly understood. In this study, we investigate isotopic fractionation of the N and O isotopes of nitrate imparted during its dissimilatory reduction by monocultures of both freshwater and marine denitrifying bacteria, with a particular focus on the relationship between the N and O isotopes. Our data reveal consistent trends with regard to the coupled N and O fractionation, trends that can provide constraints for use of the coupled nitrate N and O isotopes as a tracer of biogeochemical N cycling. In addition, the culture observations provide a basis for understanding the physiological mechanism underlying nitrate isotopic fractionation during denitrification.

## Methods

**Experimental strains**—Four strains of facultative aerobic chemo-heterotrophic denitrifying bacteria were examined: *Pseudomonas stutzeri* (American Type Culture Collection

[ATCC] 14405), *Ochrobactrum* sp. (isolated in the Delaware estuary by B. Song; Song and Ward 2003), *Paracoccus denitrificans* (ATCC 19367), and *Pseudomonas chlororaphis* f. sp. *aureofaciens* (ATCC 13985). A nondenitrifying, anoxygenic photo-heterotrophic bacterial strain was also investigated, *Rhodobacter sphaeroides* (Deutsche Sammlung von Mikroorganismen [DSM] 158). *P. stutzeri* and *Ochrobactrum* sp. are marine isolates, *P. denitrificans* and *P. chlororaphis* are soil isolates, and *R. sphaeroides* is a freshwater isolate.

The experimental strains, with the exception of *R. sphaeroides*, typify “classical” denitrifiers that respire reduced substrates, using nitrate as a terminal acceptor to fuel anaerobic growth. Respiratory nitrate reduction by denitrifiers occurs via a membrane-bound nitrate reductase (NAR), the catalytic site of which is oriented toward the cytoplasm. Respiratory denitrifiers can also express a cytoplasmic assimilatory nitrate reductase (NAS) as well as an auxiliary periplasmic nitrate reductase (NAP). The latter is not a respiratory reductase, but rather appears to be involved in redox balancing using nitrate as an ancillary oxidant to dissipate excess reductant generated during oxidative metabolism (Ellington et al. 2002); NAP is also essential for redox-poising of cyclic electron transport during photosynthetic growth of photo-heterotrophs (Gavira et al. 2002).

*R. sphaeroides* (DSM 158) was included among the experimental strains because it dissimilates nitrate exclusively with the auxiliary NAP. *R. sphaeroides* (DSM 158) is not a respiratory denitrifier in that it does not possess NAR (Gavira et al. 2002). It respire oxygen and can express the assimilatory NAS and the dissimilatory NAP during aerobic growth. This evokes the association of NAP with so-called ‘aerobic’ denitrification (Robertson and Kuenen 1990).

**Growth conditions**—Seawater medium consisted of the artificial seawater medium AQUIL (Price 1988/1989) amended with  $10\ \mu\text{mol L}^{-1}$  phosphate, between  $100\ \mu\text{mol L}^{-1}$  and  $2\ \text{mmol L}^{-1}$  nitrate,  $0.2\ \text{g L}^{-1}$  casein

hydrolysate, and 0.2 g L<sup>-1</sup> bactopeptone. The medium was sterilized by microwaving (Keller et al. 1988), then supplemented with filter-sterilized AQUIL trace metals and f/2 vitamins. Freshwater medium consisted of salt concentrations specified in RCV medium (Weaver et al. 1975) supplemented with 0.2 g L<sup>-1</sup> casein hydrolysate and 0.2 g L<sup>-1</sup> bactopeptone. Autoclaved RCV medium was amended with AQUIL trace metals, f/2 vitamins, and nitrate additions, as specified above.

Cells were grown in batch culture at room temperature. Cultures were initiated from frozen stocks and acclimated aerobically to experimental media for 10 generations. Overnight cultures were then inoculated in 250-mL, opaque, tri-laminate, polyethylene-lined, TEDLAR™ gas-tight bags. These had been previously rinsed with 10% HCl and milli-Q water.

Freshwater and seawater isolates were grown in their respective media. To investigate whether strains fractionate nitrate isotopes differently in freshwater than in seawater, the freshwater and soil isolates *P. denitrificans*, *P. chlororaphis*, and *R. sphaeroides* were also cultured in seawater medium, as they proved to be halo-tolerant. Growth of all cultures was monitored by measuring the transient accumulation of nitrite in the culture bags or by measuring the disappearance of nitrate. Nitrite was quantified colorimetrically by reaction with Greiss reagents (Parsons et al. 1984). Nitrate was measured by conversion to NO in a hot vanadium(III) solution followed by chemiluminescence detection (Braman and Hendrix 1989) with an Antek 1750 nitrate/nitrite analyzer. Under the conditions used, chemiluminescence detection simultaneously measured both nitrate and nitrite in a sample; to measure nitrate without the interference of nitrite, we trapped nitrite with sulfanilamide prior to sample injection in the vanadium(III) solution.

Subsamples (20 mL) of growing culture were sequentially extracted from the bags for isotope analysis of the nitrate as it was progressively depleted from cultures. The culture subsamples were stored in acid-washed polypropylene bottles and frozen immediately in a -10°C freezer.

*Nitrate isotope analyses*—Prior to nitrate N and O isotope analyses, nitrite in the culture samples was removed with ascorbate (Granger et al. 2006), because nitrite interferes with nitrate isotope analyses. Thawed samples were first divided into duplicates in sealed serum vials. The vials were heated to ca. 90°C for 10 min on a hot plate in order to inhibit any bacterial activity that may have remained after freezing of the samples. Nitrite was then removed by adding 10 mmol L<sup>-1</sup> ascorbate while purging with an inert gas. Duplicates were processed in separate batches to eliminate potential systematic error associated with individual batch processing.

We noticed that during prolonged sample storage (i.e., a few months), nitrite concentrations decreased in some samples; nitrite can be unstable, even when frozen (Thayer 1970). This resulted in N and O isotopic shifts at lower nitrate concentrations that were likely due to the spontaneous decomposition of nitrite to nitric oxide and reoxidation of nitric oxide to nitrate (Granger et al.

2006). Consequently, we proceeded to remove nitrite from samples within a few weeks of their collection. Isotope ratios measured for experiments that had been stored for approximately 6 months or more prior to nitrite removal showed sporadic and haphazard isotope behavior at lower nitrate concentrations when the proportion of nitrite was relatively high. Data generated from these experiments were discarded. However, a bias remains possible in some experiments. Work that involves nitrite can prove problematic in many analytical contexts, and methodology to minimize its interference is evolving (Casciotti et al. 2007).

The <sup>15</sup>N:<sup>14</sup>N and <sup>18</sup>O:<sup>16</sup>O of nitrate were determined following the denitrifier method (Sigman et al. 2001; Casciotti et al. 2002). This method involves the quantitative conversion of nitrate (and nitrite) in a sample to nitrous oxide (N<sub>2</sub>O) gas by denitrifying bacteria that lack the N<sub>2</sub>O reductase enzyme and thus do not dismutate N<sub>2</sub>O to dinitrogen gas. The N and O isotopic composition of the N<sub>2</sub>O gas analyte is then measured on a gas chromatograph–isotope ratio mass spectrometer. Isotope ratios are reported using the delta (δ) notation in units of per mil (‰), thus:

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

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

The <sup>15</sup>N:<sup>14</sup>N reference is N<sub>2</sub> in air, and the <sup>18</sup>O:<sup>16</sup>O reference is Vienna standard mean ocean water (VSMOW). Individual analyses were referenced to injections of N<sub>2</sub>O from a pure gas cylinder and then standardized through comparison to the international potassium nitrate reference materials International Atomic Energy Agency NO<sub>3</sub><sup>-</sup> (IAEA-N3) with an assigned δ<sup>15</sup>N of +4.7, vs. atmospheric N<sub>2</sub> (Gonfiantini et al. 1995), and a most recently reported δ<sup>18</sup>O of 25.6‰, vs. VSMOW (Böhlke et al. 2003). The size of the culture blank was determined by running a prepared vial to which no sample was added. An O isotope correction is also required for exchange of the oxygen atoms with water during reduction of nitrate to N<sub>2</sub>O (Casciotti et al. 2002). In initial experiments, exchange was calculated for each batch of measurements from the slope of the regression between δ<sup>18</sup>O of IAEA-N3 in normal vs. <sup>18</sup>O-enriched water (δ<sub>H<sub>2</sub>O</sub> = 300‰; Casciotti et al. 2002). Later it was calculated by comparing the observed and reported δ<sup>18</sup>O difference between IAEA-N3 and the nitrate isotopic reference material United States Geological Survey 34 (USGS-34; δ<sup>18</sup>O = -27.9‰; Böhlke et al. 2003). Use of either correction scheme did not change the reported values appreciably.

The N and O isotopic measurements of roughly 10% of the samples were replicated within a day's batch of analyses, yielding standard deviations of 0.2‰ for δ<sup>15</sup>N and 0.5‰ for δ<sup>18</sup>O. As mentioned above, the majority of the samples were also processed in duplicates for nitrite

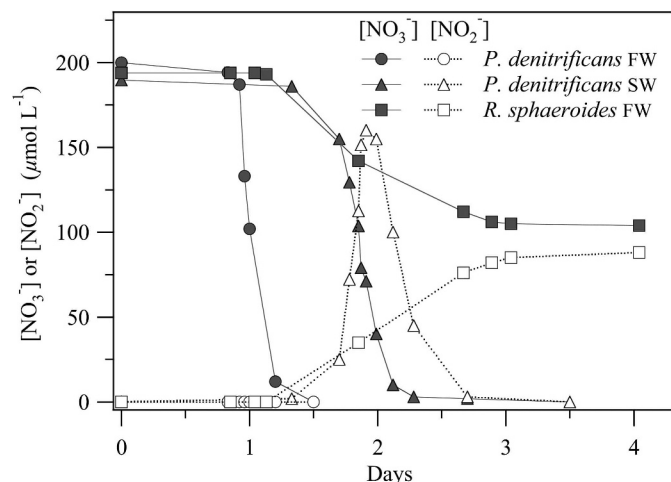


Fig. 1. Changes in the nitrate and nitrite concentrations from the time of inoculation in growing cultures of *P. denitrificans* in freshwater (FW) medium, *P. denitrificans* in seawater (SW) medium, and *R. sphaeroides* in freshwater medium.

removal. Reproducibility among duplicate nitrite removals of a given sample was 0.4‰ for  $\delta^{15}\text{N}$  and 0.8‰ for  $\delta^{18}\text{O}$ .

To derive estimates of the N and O isotope effects imparted on nitrate ( $^{15}\epsilon$  and  $^{18}\epsilon$ ) during growth, the nitrate  $\delta^{15}\text{N}$  and  $\delta^{18}\text{O}$  measurements were fit to the following linear equations (Mariotti et al. 1981):

$$\ln(\delta^{15}\text{N} + 1) = \ln(\delta^{15}\text{N}_{\text{initial}} + 1) + (^{15}\epsilon/1000)\ln(f) \quad (3)$$

$$\ln(\delta^{18}\text{O} + 1) = \ln(\delta^{18}\text{O}_{\text{initial}} + 1) + (^{18}\epsilon/1000)\ln(f) \quad (4)$$

where  $f = [\text{NO}_3^-]/[\text{NO}_3^-]_{\text{initial}}$ . Regression of  $\ln(\delta^{15}\text{N} + 1)$  or  $\ln(\delta^{18}\text{O} + 1)$  on  $\ln(f)$  yields respective slopes of  $^{15}\epsilon/1000$  or  $^{18}\epsilon/1000$ .

A simplified linearization of the Rayleigh model was used to plot the isotope ratio measurements. Namely, the  $\delta^{15}\text{N}$  of nitrate was plotted on  $\ln([\text{NO}_3^-])$  and  $\ln(f)$  according to the more intuitive (but less accurate) linear derivation (Mariotti et al. 1981), where the slope of the line approximates  $^{15}\epsilon$ :

$$\delta^{15}\text{N} = \delta^{15}\text{N}_{\text{initial}} + ^{15}\epsilon(\ln([\text{NO}_3^-])) \quad (5a)$$

$$\delta^{15}\text{N} = \delta^{15}\text{N}_{\text{initial}} + ^{15}\epsilon(\ln(f)) \quad (5b)$$

## Results

*Growth of the denitrifier cultures*—The respiratory denitrifiers fueled their initial growth using the  $\text{O}_2$  in the medium for respiration. As cell densities increased, the  $\text{O}_2$  concentration presumably decreased, and denitrifying cells switched to nitrate respiration. During growth, most of the respiratory denitrifier cultures showed a stoichiometric accumulation of nitrite as nitrate was depleted from the cultures, followed by depletion of nitrite from the culture medium once nitrate had been consumed (Fig. 1). However, *Ochrobactrum* sp., as well as *P. denitrificans* grown in

freshwater medium (Fig. 1), generally showed little to no nitrite accumulation during nitrate consumption, implying that any nitrite generated was respired concomitantly with nitrate.

*R. sphaeroides* grew aerobically in the opaque culture bags using the oxygen dissolved in the medium and reduced nitrate to nitrite during aerobic growth. However, in contrast to the other experimental strains, *R. sphaeroides* ceased growing following the depletion of oxygen from the bags, and it rarely consumed all ambient nitrate, presumably because it was depleted from the cultures more rapidly than nitrate during growth (Fig. 1). Indeed, deliberate addition of air to the spent cultures induced further nitrate reduction (data not shown). Nitrate consumption during heterotrophic growth of *R. sphaeroides* was comparatively slow and most pronounced during the stationary phase, as observed by others (Gavira et al. 2002). Moreover, none of the nitrite produced by *R. sphaeroides* was consumed, because this strain does not express a respiratory periplasmic nitrite reductase (Gavira et al. 2002).

*Nitrate N and O isotope fractionation by ‘respiratory’ denitrifiers*—The nitrate N and O isotope ratios among the four respiratory denitrifiers—*P. stutzeri*, *Ochrobactrum* sp., *P. chlororaphis*, and *P. denitrificans*—generally conformed to a linear relationship of  $\delta^{15}\text{N}$  or  $\delta^{18}\text{O}$  against the natural logarithm of the fraction of nitrate remaining, as predicted by the Rayleigh model (Eq. 5). The respective regression coefficients yield estimates of the N and the O isotope effects associated with the respiration of nitrate (Eqs. 3 and 4; Table 2). However, in cases described below, a characteristic asymptote of the isotope ratios developed at lower nitrate concentrations for both N and O (Fig. 2). The samples that showed this behavior were not included in the regression analyses to derive respective isotope effects. The  $r^2$  values of the resulting linear regressions were consistently greater than 0.97 (analyses not shown).

Nitrate N isotope effects ( $^{15}\epsilon$ ) observed among the respiratory denitrifiers spanned a broad range, between 5‰ and 25‰, although the majority of these estimates were above 15‰ (Table 2). Similarly, corresponding  $^{18}\epsilon$  estimates ranged between 5‰ and 23‰, and most of these exceeded 15‰. These variations did not correspond to differences specific to given strains. For example, replicate cultures of *P. stutzeri* (Table 2, experiments 28A,B) showed relatively low N isotope effects of 5‰ and 10‰ in one set of experiments but showed N isotope effects of 18‰ and 20‰ in another set of experiments conducted at a later date (Table 2, experiments 36A,B). Moreover, variations in the isotope effects could not be ascribed to differences in medium salinity, as growth of *P. denitrificans* and *P. chlororaphis* in seawater vs. freshwater media resulted in no distinct difference for  $^{15}\epsilon$  or  $^{18}\epsilon$ . Admittedly, we only have data for a single culture of *P. chlororaphis* in freshwater medium (Table 2), such that differences in isotope effects due to medium salinity could become apparent given more replicates with this strain.

A distinct feature in some of the cultures is the apparent asymptotic behavior of the Rayleigh plots at lower nitrate concentrations (Fig. 2a). *P. stutzeri* (experiment 28B)

Table 2. Nitrate nitrogen (N) and oxygen (O) isotope effects ( $^{15}\epsilon$  and  $^{18}\epsilon$ , respectively) computed for individual experiments (Exp.) with five denitrifying bacterial strains and corresponding  $\Delta\delta^{18}\text{O}:\Delta\delta^{15}\text{N}$  relationships.  $^{15}\epsilon$  and  $^{18}\epsilon$  were derived from the slopes of individual regression analyses (Eqs. 3 and 4) and are reported  $\pm 1$  standard deviation associated with respective slopes. Similarly,  $\Delta\delta^{18}\text{O}:\Delta\delta^{15}\text{N}$  refers to the slope of the linear regression of  $\delta^{18}\text{O}$  vs. its corresponding  $\delta^{15}\text{N}$  measurement,  $\pm 1$  standard deviation associated with the slope. Note that some  $\delta^{15}\text{N}$  vs  $\ln f$  trends were no longer linear at relatively low nitrate concentrations (Fig. 2), such that asymptotic points were omitted in related regression analyses. Regression analyses included measurements from at least three and up to seven distinct samples. SW = seawater, FW = freshwater.

Strain	Exp	Medium	$[\text{NO}_3^-]_{\text{initial}}$ ( $\mu\text{mol L}^{-1}$ )	$^{15}\epsilon$ (‰)	$^{18}\epsilon$ (‰)	$\Delta\delta^{18}\text{O}:\Delta\delta^{15}\text{N}$
<i>Ochrobactrum</i> sp.	26B	SW	101	22.9 $\pm$ 1.7	21.1 $\pm$ 1.6	0.94 $\pm$ 0.02
	33A	SW	2779	6.7 $\pm$ 0.7	6.8 $\pm$ 0.4	1.02 $\pm$ 0.06
	37A	SW	510	21.8 $\pm$ 2.9	20.2 $\pm$ 3.3	0.96 $\pm$ 0.03
	37B	SW	500	22.8 $\pm$ 0.8	22.8 $\pm$ 1.9	1.02 $\pm$ 0.03
	40A	SW	315	15.0 $\pm$ 0.3	14.4 $\pm$ 0.6	0.98 $\pm$ 0.01
	40B	SW	315	17.6 $\pm$ 0.1	17.2 $\pm$ 0.2	1.00 $\pm$ 0.01
<i>Pseudomonas chlororaphis</i>	19A	SW	208	20.5 $\pm$ 1.5	19.7 $\pm$ 0.4	0.94 $\pm$ 0.02
	19B	SW	166	23.0 $\pm$ 1.2	21.1 $\pm$ 1.1	0.97 $\pm$ 0.01
	21A	SW	256	20.9 $\pm$ 0.9	18.9 $\pm$ 0.1	0.91 $\pm$ 0.03
	21B	SW	261	22.5 $\pm$ 1.7	20.8 $\pm$ 0.9	0.94 $\pm$ 0.04
	48A	FW	310	16.9 $\pm$ 0.5	16.4 $\pm$ 0.4	0.99 $\pm$ 0.01
<i>Paracoccus denitrificans</i>	7A	SW	202	17.6 $\pm$ 1.5	16.5 $\pm$ 1.4	0.95 $\pm$ 0.04
	7B	SW	180	20.0 $\pm$ 2.1	17.7 $\pm$ 2.9	0.92 $\pm$ 0.02
	39A	SW	315	24.8 $\pm$ 0.4	22.6 $\pm$ 0.4	0.92 $\pm$ 0.01
	39B	SW	310	26.6 $\pm$ 0.5	22.5 $\pm$ 0.9	0.91 $\pm$ 0.05
	30A	FW	95	23.5 $\pm$ 0.6	20.7 $\pm$ 0.3	0.90 $\pm$ 0.02
<i>Pseudomonas stutzeri</i>	38B	FW	314	18.8 $\pm$ 0.3	17.9 $\pm$ 0.3	0.97 $\pm$ 0.01
	28A	SW	89	9.7 $\pm$ 0.2	8.1 $\pm$ 0.2	0.86 $\pm$ 0.04
	28B	SW	85	5.4 $\pm$ 0.3	4.8 $\pm$ 0.1	0.92 $\pm$ 0.02
	36A	SW	500	19.7 $\pm$ 1.3	17.7 $\pm$ 1.1	0.88 $\pm$ 0.02
<i>Rhodobacter sphaeroides</i>	36B	SW	513	17.7 $\pm$ 1.8	15.6 $\pm$ 1.7	0.91 $\pm$ 0.02
	27A	FW	100	12.6 $\pm$ 0.2	7.9 $\pm$ 0.4	0.60 $\pm$ 0.02
	27B	FW	109	13.7 $\pm$ 0.2	8.6 $\pm$ 0.1	0.63 $\pm$ 0.01
	41A	FW	303	14.7 $\pm$ 0.3	8.7 $\pm$ 1.0	0.60 $\pm$ 0.02
	41B	FW	301	14.5 $\pm$ 0.1	8.6 $\pm$ 0.3	0.61 $\pm$ 0.02
	49A	FW	325	18.1 $\pm$ 0.2	10.3 $\pm$ 0.2	0.59 $\pm$ 0.03
	49B	FW	325	16.0 $\pm$ 0.2	8.9 $\pm$ 0.1	0.57 $\pm$ 0.00
	49C	FW	290	15.7 $\pm$ 0.4	9.5 $\pm$ 0.1	0.61 $\pm$ 0.00
	49D	FW	328	14.8 $\pm$ 2.7	9.7 $\pm$ 2.3	0.68 $\pm$ 0.04
	50A	SW	295	15.9 $\pm$ 0.2	8.9 $\pm$ 0.2	0.58 $\pm$ 0.03
50B	SW	285	19.9 $\pm$ 0.2	13.1 $\pm$ 0.2	0.66 $\pm$ 0.06	

showed no further increase in nitrate  $\delta^{15}\text{N}$  at concentrations lower than 3  $\mu\text{mol L}^{-1}$ . In the same figure, a similar deviation of  $^{15}\epsilon$  from linearity is discernable for *P. chlororaphis* (experiment 19B) at 6  $\mu\text{mol L}^{-1}$  nitrate. This trend is not visually apparent for all cultures that were sampled at lower nitrate concentrations, yet this may result from the lack of sampling intervals at intermediate concentrations for some cultures. Consequently, estimates of isotope effects for two cultures may be somewhat underestimated, as these regression coefficients weighed heavily on samples with a relatively low nitrate concentration (i.e., *Ochrobactrum* sp. experiment 40A and *P. denitrificans* experiment 7A).

While  $^{18}\epsilon$  and  $^{15}\epsilon$  often differed between experiments within and among strains, all estimates of  $^{18}\epsilon$  were similar to their corresponding  $^{15}\epsilon$  estimates. Linear regressions of  $\delta^{18}\text{O}$  on the  $\delta^{15}\text{N}$  of corresponding samples for individual experiments (including the asymptotic N and O isotope ratios) yielded slopes that were consistently close to 1, although most commonly slightly below it (Table 2). The lowest slopes observed were among the four *P. stutzeri*

cultures ( $\sim 0.89$ ), while *Ochrobactrum* sp. consistently showed higher  $\Delta\delta^{18}\text{O}:\Delta\delta^{15}\text{N}$  trajectories, averaging 0.98 for six cultures. Overall, the average  $\Delta\delta^{18}\text{O}:\Delta\delta^{15}\text{N}$  ( $\pm 1 \sigma$ ) for all measurements among the four respiratory strains was  $0.96 \pm 0.01$  (Fig. 3)—thus,  $^{18}\epsilon$  and  $^{15}\epsilon$  were nearly equivalent.

*Nitrate N and O isotope fractionation during aerobic denitrification by R. sphaeroides*—The photo-heterotroph *R. sphaeroides* covered a narrower range of magnitudes in  $^{15}\epsilon$  and  $^{18}\epsilon$  than the sum of the respiratory denitrifiers. Values for  $^{15}\epsilon$  ranged between 13‰ and 20‰ (Table 2), showing a modal value of  $\sim 15$ ‰ (Fig. 4). Estimates of  $^{18}\epsilon$  ranged between 8‰ and 13‰ (Table 2), with a modal value of  $\sim 9$ ‰. Like the respiratory denitrifiers, growth of *R. sphaeroides* in seawater (for two cultures only) showed no consistent difference in isotope effects compared to freshwater cultures.

As observed above, nitrate isotope fractionation by *R. sphaeroides* also seemed to asymptote for a single culture that reached the lowest observed nitrate concentration

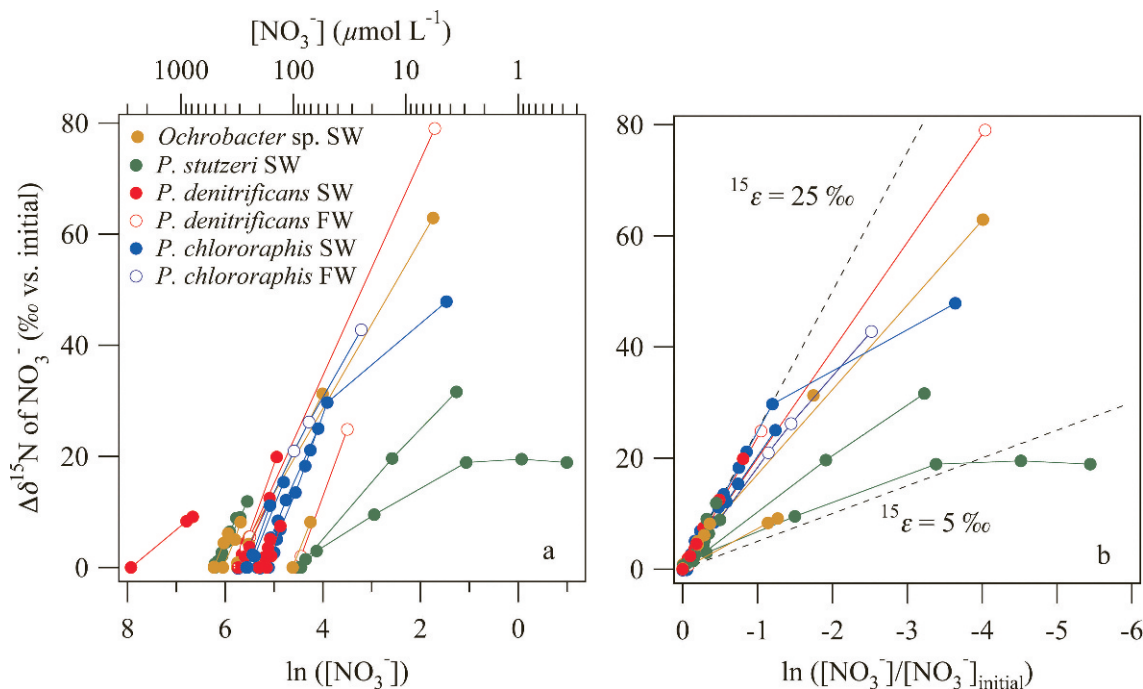


Fig. 2. Rayleigh plots of the change in the N isotopic composition of nitrate ( $\delta^{15}\text{N}$ ) as a function of the natural logarithm of nitrate consumption for four strains of respiratory denitrifiers grown in freshwater (FW) and seawater (SW) media. (a)  $\delta^{15}\text{N}$  is plotted over the  $\ln$  of nitrate concentrations (reverse scale) to highlight the asymptotic behavior of nitrate N isotopic fractionation at lower nitrate concentrations. (b)  $\delta^{15}\text{N}$  is plotted over the  $\ln$  of fractional nitrate use (reverse scale) to show the range in N isotope effects observed for the experimental strains.

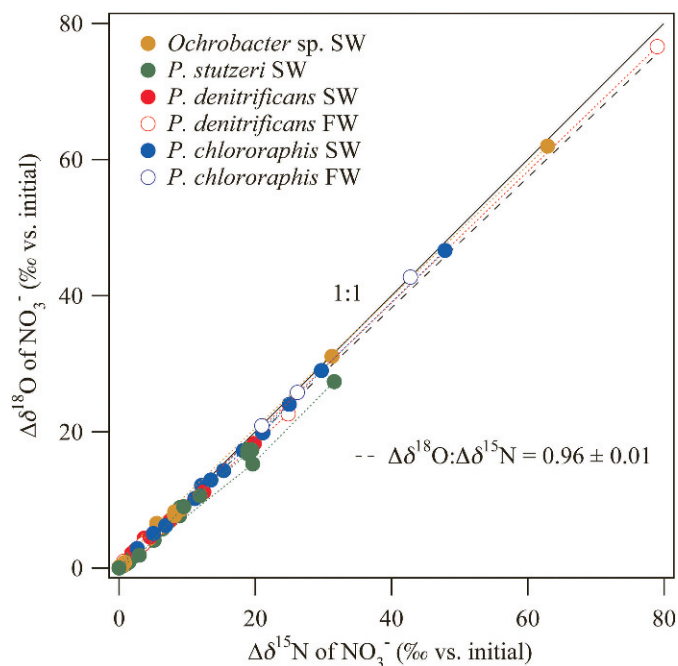


Fig. 3. The  $\delta^{18}\text{O}$  of nitrate plotted against the corresponding  $\delta^{15}\text{N}$  of nitrate for cultures of four strains of respiratory denitrifiers grown in freshwater and seawater media. The mean slope of all measurements yields a value ( $\pm 1$  standard deviation) of  $0.96 \pm 0.01$ .

(Table 2, experiment 27A; Fig. 4a). Inflection of the Rayleigh slope was apparent between 12 and 30  $\mu\text{mol L}^{-1}$  (Fig. 4a). We are uncertain as to the significance of this trend because it derives from a single nitrate sample.

With regard to  $\Delta\delta^{18}\text{O}:\Delta\delta^{15}\text{N}$ , *R. sphaeroides* was clearly distinct from the respiratory denitrifiers in that its average  $\Delta\delta^{18}\text{O}:\Delta\delta^{15}\text{N}$  ( $\pm 1\sigma$ ) was  $0.62 \pm 0.02$ , in contrast to 0.96 (Fig. 5; Table 2).

## Discussion

*Nitrate N and O isotope fractionation by respiratory denitrifiers*—The observations in this study demonstrate that respiratory denitrification imparts nearly identical isotope effects on the N and O isotopes of nitrate ( $^{18}\epsilon \sim ^{15}\epsilon$ ), consistent with the interpretation of ocean field data (Sigman et al. 2005). In contrast to the constant  $\Delta\delta^{18}\text{O}:\Delta\delta^{15}\text{N}$ , the magnitude of the N (and O) isotope effects varied significantly among cultures, highlighting the potential for plasticity of the isotope effect amplitudes.

Heavy isotope fractionation on nitrate results from the cumulative discrimination associated with individual steps in denitrification, through to the first irreversible step (Fig. 6). Thus, to determine the origin of the isotope effect, we consider not only the potential for isotope discrimination at a given step but also the potential for chemical or physical reversibility of the steps before it. In sequence, these steps are (1) the diffusion of nitrate to the cell surface and into the periplasm, (2) the active transport of nitrate

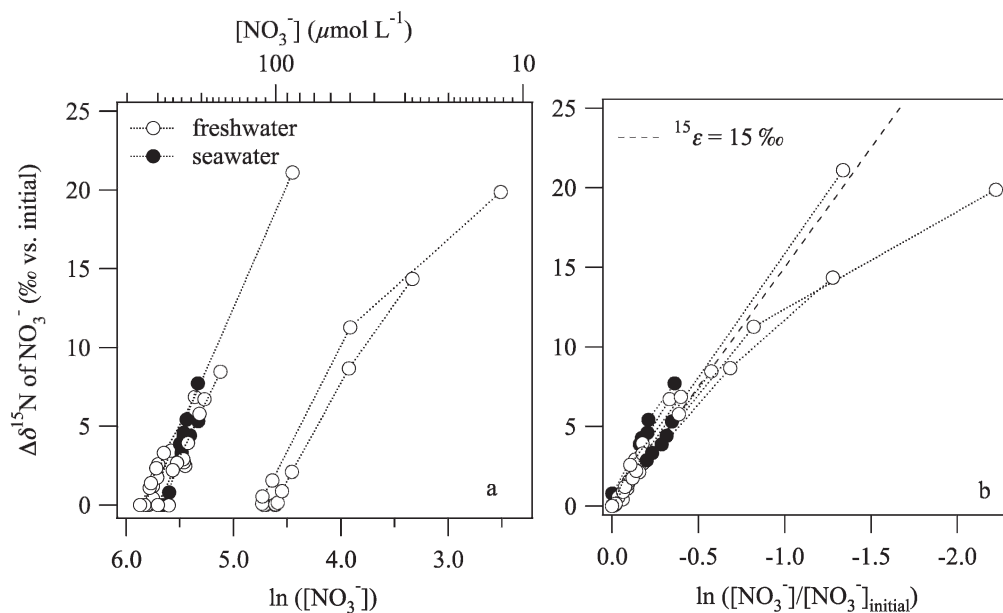
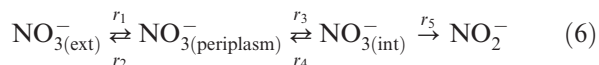


Fig. 4. Rayleigh plots of the change in the N isotopic composition of nitrate ( $\delta^{15}\text{N}$ ) as a function of the natural logarithm of nitrate use for freshwater and seawater cultures of the photo-heterotroph *R. sphaeroides*. (a)  $\delta^{15}\text{N}$  is plotted over the  $\ln$  of nitrate concentrations to highlight the asymptotic behavior of nitrate N isotopic fractionation at lower nitrate concentrations. (b)  $\delta^{15}\text{N}$  is plotted over the  $\ln$  of fractional nitrate use (reverse scale) to show the cluster in N isotope effects observed for *R. sphaeroides*.

across the inner bacterial membrane, and (3) the enzymatic reduction of intracellular nitrate by the dissimilatory NAR or the assimilatory NAS. Enzymatic bond breakage represents the last step during which an isotope effect can be imparted on nitrate, since the nitrite product does not revert back to nitrate. This scheme is summarized as



Transport of material from one state to the next is described by respective rates,  $r_1$ ,  $r_2$ ,  $r_3$ ,  $r_4$ , and  $r_5$ . For example, the rate of conversion of  $\text{NO}_3^-(\text{ext})$  to  $\text{NO}_3^-(\text{periplasm})$  is given by  $r_1$ , the forward rate for the diffusion of  $\text{NO}_3^-(\text{ext})$ . The dissimilation of nitrate by the auxiliary NAP, which involves the diffusion of nitrate into the periplasm followed by bond breakage at the site of the periplasmic NAP, is not considered in Eq. 6. Its effect on isotopic discrimination during respiratory denitrification will be considered later.

Considering the conversion of  $\text{NO}_3^-(\text{ext})$  to  $\text{NO}_3^-(\text{periplasm})$ , a large contribution of nitrate diffusion to the observed N and O isotope effects is unlikely for two reasons. First, evidence indicates that nitrate in solution is solvated, such that the mass difference between nitrate isotopologues is relatively small. Consequently, the isotope effects associated with nitrate diffusion are likely negligible (Mariotti et al. 1988). Second, the diffusive flux of nitrate to the cell surface is orders of magnitude faster than the ambient rate of transport by the bacterial cell at biologically relevant nitrate concentrations ( $r_1 \gg r_3$ ) (Pasciak and Gavis 1974). Hence, gross diffusion to and from the periplasm are nearly equal ( $r_1 = r_2$ ), such that any kinetic fractionations imposed by these transport terms would nearly cancel one another.

Next, the active transport of nitrate from the periplasm into the cell,  $r_3$ , arguably has a limited effect on the bonding environment of the nitrate molecule, such that, according to transition state theory, any associated isotope effect should be relatively small (Melander and Saunders 1980). Indeed, the limited impact of nitrate transport on isotope fractionation has been noted previously in the context of nitrate assimilation. Mariotti et al. (1982) observed that nitrate was no longer fractionated by seedlings of Pearl Millet when external nitrate was low and transport became the rate-determining step in nitrate assimilation, at which point isotopic discrimination should approach that associated with nitrate uptake into the plant. Wada and Hattori (1978) observed a range of N isotope effects for diatom monocultures subjected to different light intensities and measured an isotope effect as low as 0.5‰ at the highest experimental irradiance, from which they concluded that a transport isotope effect could be no greater than this value. Similarly, Waser et al. (1988a) and Needoba et al. (2003) measured N isotope enrichment during nitrate assimilation by numerous species of unicellular algae and observed N isotope effects as low as 2‰. These low values could also be construed as an upper limit on N isotope discrimination by nitrate transport. In addition, Waser et al. (1988b) detected no N isotopic enrichment during nitrite assimilation by a diatom strain, indicating that cellular transport of nitrite is not a fractionating process; by extension, transport of nitrate is not likely to impart a large N isotopic enrichment. Thus, physico-chemical constraints on kinetic isotope discrimination, as well as experimental observations, indicate that transport alone cannot account for a substantial part of the observed isotope fractionation on nitrate when the isotope effect is high ( $\sim 25\%$ ). At the same time, a modest isotope

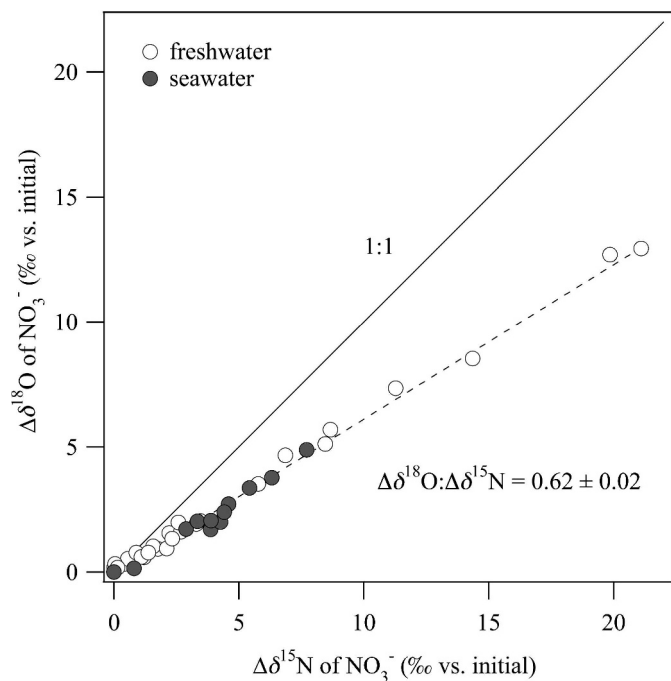


Fig. 5. The  $\delta^{18}\text{O}$  of nitrate plotted against the corresponding  $\delta^{15}\text{N}$  of nitrate for cultures *R. sphaeroides* grown in freshwater and seawater media. The mean slope of all measurements yields a value ( $\pm 1$  standard deviation) of  $0.62 \pm 0.02$ .

effect (say  $\leq 5\%$ ) associated with transport cannot be ruled out for our cultures.

In contrast to transport, a large isotope effect associated with the enzymatic rupture of a nitrate N–O bond is expected (Bigeleisen 1949) and observed (Ledgard et al. 1985; Schmidt and Medina 1991). In vitro measurements of nitrate N isotope fractionation reported for eukaryotic assimilatory nitrate reductases show relatively high intrinsic N isotope effects, ranging between 15‰ for spinach (Ledgard et al. 1985) to 30‰ for *Chlorella* and maize (Schmidt and Medina 1991). The sizeable N isotope effects associated with nitrate reductase activity thus point to enzymatic catalysis as the most plausible cause of heavy isotope enrichment of nitrate during denitrification. As explained below, our observations appear consistent with this.

Up to three distinct nitrate reductases can be active during growth of respiratory denitrifiers, making each a potential candidate for imparting isotope discrimination on nitrate. The activity of membrane-bound NAR is ostensibly high during anaerobic growth, identifying NAR as a likely driver of the isotope enrichment during denitrification. The cytosolic assimilatory NAS, in contrast, is probably not important in fractionation, because NAS expression is repressed in the presence of ammonia (Warneckeberz and Friedrich 1993); ammonia is a sizeable constituent bactopectone and casein hydrolysate in our growth media, and is further generated by catabolism of amino acids during bacterial growth. The activity of the periplasmic NAP during anaerobic growth, which will be addressed below, is probably considerably lower than that of the respiratory NAR, because NAP is not associated

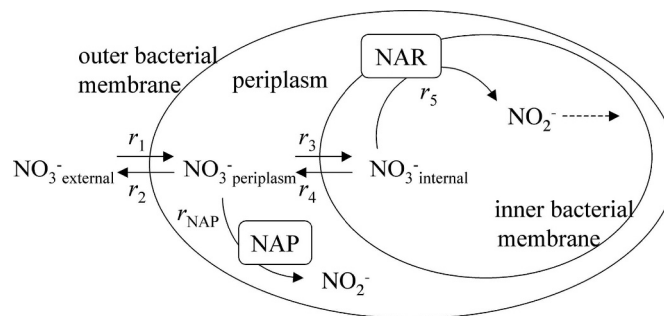


Fig. 6. A diagram of nitrate metabolism in a denitrifying cell. Transformations of nitrate from one state to another are described by respective rates, from  $r_1$  to  $r_5$ , as in Eq. 6. External nitrate diffuses into and from the periplasm ( $r_1$  and  $r_2$ ) through the pores of the outer bacterial membrane. Nitrate in the periplasm can be actively transported into the cell ( $r_3$ ), where it undergoes reduction to nitrite catalyzed by the respiratory membrane-bound nitrate reductase NAR ( $r_5$ ). Nitrate inside the cell may also be subject to efflux back into the periplasm ( $r_4$ ). The nitrite produced in the cell's interior is actively exported out of the cell into the periplasm, where it can serve to fuel respiration by the soluble periplasmic nitrite reductase (NiR) (not shown). Nitrate in the periplasm can also be reduced by the soluble periplasmic NAP ( $r_{\text{NAP}}$ ) during aerobic growth, and perhaps even during anaerobic growth of denitrifiers (Bedzyk et al. 1999).

with energy production. Since NAR catalyzes the bulk of cellular nitrate reduction, it is presumably the dominant driver of the majority of the observed isotope effect.

While we have identified NAR as the likely dominant driver of fractionation, the extent to which the isotope enrichment from bond breakage is propagated to external nitrate is ultimately controlled by the reversibility of nitrate transport ( $r_3$  vs.  $r_4$ ). If the enzymatic isotope effect is to be expressed beyond the internal pool, intracellular nitrate must be subject to a reverse metabolic flow ( $r_4 \neq 0$ ), wherein nitrate reduction inside the cell is coupled with some rate of nitrate efflux back into the medium. Put another way, if internal nitrate were perfectly sequestered and had no metabolic fate except to be carried through to dissimilation by nitrate reductase ( $r_4 = 0$ ), then nitrate uptake would represent the first irreversible step in denitrification, such that isotope fractionation associated with enzymatic bond breakage would not be observed in external nitrate. Thus, given that enzymatic reduction is the only step in denitrification that can impart a large ( $\gg 5\%$ ) isotope effect, the heavy isotope enrichments observed for external nitrate in many of our experiments indicate a significant efflux term for internal nitrate. Accordingly, the  $\Delta\delta^{18}\text{O}:\Delta\delta^{15}\text{N}$  of  $\sim 1$  associated with the highest amplitude isotope effects is ostensibly that intrinsic to NAR activity. By extension, to the degree that different physiological processes are not expected to show the same  $\Delta\delta^{18}\text{O}:\Delta\delta^{15}\text{N}$  of  $\sim 1$ , the invariance of this O-to-N isotope coupling, despite the large amplitude differences observed in isotope effects among experiments, implies that isotope discrimination by NAR is, indeed, the dominant source of fractionation in all experiments, with any isotope discrimination by transport being negligible. A modest transport isotope effect (e.g., 5‰ or less) that was to impart a

different  $\Delta\delta^{18}\text{O}:\Delta\delta^{15}\text{N}$  on nitrate would be manifested as a departure from the observed  $\Delta\delta^{18}\text{O}:\Delta\delta^{15}\text{N}$  relationship that should become increasingly evident in experiments showing lower amplitude isotope effects (i.e., cultures sustaining less propagation of the enzymatic isotope effect by cellular efflux). Such a trend is not apparent in our data, as the  $\Delta\delta^{18}\text{O}:\Delta\delta^{15}\text{N}$  of  $\sim 1$  is maintained at lower measured isotope effects.

Remarkably, the  $\Delta\delta^{18}\text{O}:\Delta\delta^{15}\text{N}$  of the respiratory denitrifiers was not only invariant among experimental cultures, but it was also maintained within cultures that showed curvature in isotope effect amplitude at lower nitrate concentrations. The curvature of the nitrate  $\delta^{15}\text{N}$  and  $\delta^{18}\text{O}$  is best explained as a reduction of cellular nitrate N and O isotope effects due to a decrease in nitrate efflux as a fraction of the gross nitrate influx. As nitrate reaches subsaturating levels with respect to the transporter(s), the rate of nitrate transport ( $r_3$ ) approaches proportionality with external nitrate concentrations, and the nitrate supply to the internal pool becomes insufficient to maintain saturated enzymatic activity. Transport thus begins to limit the rate of nitrate reduction and ultimately becomes the first irreversible step (as  $r_4 \rightarrow 0$ ) in the denitrification pathway, at which point any fractionation of the N and O isotopes in external nitrate would be driven solely by nitrate uptake. That the simultaneous inflection of N and O isotope discrimination is apparent in cultures in the range of  $3 \mu\text{mol L}^{-1}$  to  $6 \mu\text{mol L}^{-1}$  nitrate is consistent with a half-saturation constant for nitrate uptake kinetics ( $K_M$ ) of  $\sim 5 \mu\text{mol L}^{-1}$ , determined for *P. denitrificans* grown in batch cultures (Parsonage et al. 1985). If nitrate transport was imparting substantial isotope discrimination on nitrate, and if the associated  $^{18}\text{O}:\text{N}$  was different than 1, than these transport isotope effects would continue to be expressed even as the rate of transport became proportional to nitrate concentrations; fractionation of the N and O isotopes would not equivalently approach zero when transport became the rate-limiting step in denitrification. Yet, in culture experiment 28B (Fig. 2a; *P. stutzeri*), the last three samples of the experiment indicate no continued increase in either the  $\delta^{15}\text{N}$  or  $\delta^{18}\text{O}$  of external nitrate as the latter is depleted 10-fold from  $3 \mu\text{mol L}^{-1}$  to  $0.3 \mu\text{mol L}^{-1}$ , and the N and O isotopes do not deviate from 1:1 behavior as they approach that asymptote. Thus, we conclude that nitrate transport does not contribute measurably to isotopic discrimination among respiratory denitrifiers and that the isotopic enrichment in external nitrate is driven by internal NAR activity.

The conceptual model outlined here is analogous to the fractionation mechanism recognized for nitrate assimilation by unicellular prokaryotic and eukaryotic algae (Shearer et al. 1991; Granger et al. 2004; Needoba et al. 2004), as well as that in higher plants (Mariotti et al. 1982), in which heavy isotope enrichment on nitrate occurs internally through the activity of the assimilatory nitrate reductase and is propagated to external nitrate via cellular nitrate efflux. Similarly, in a heterotrophic marine bacterium, Hoch et al. (1992) invoked cellular efflux of internal ammonium to explain observations of heavy N isotope enrichment in extracellular ammonium.

Concurrent evidence that the distinct  $\Delta\delta^{18}\text{O}:\Delta\delta^{15}\text{N}$  of  $\sim 1$  is imprinted on nitrate by the activity of NAR is afforded by observations of a similar  $\Delta\delta^{18}\text{O}:\Delta\delta^{15}\text{N}$  signature of  $\sim 1$  during nitrate assimilation among numerous cultures of eukaryotic unicellular algae, for which the invariant coupling is imparted by the activity of the assimilatory nitrate reductase (Granger et al. 2004). But, in contrast to unicellular algae, the inflection in the Rayleigh plots seen here for denitrifiers was not observed among cultures of marine eukaryotic phytoplankton (Granger et al. 2004). This likely reflects a lower half-saturation constant for nitrate uptake of the marine eukaryotic algae, which is generally measured at  $<1 \mu\text{mol L}^{-1}$  (Eppley et al. 1969). Given the exponential growth of the algal cultures, nitrate concentrations in that range were not captured by the samples collected during nitrate assimilation experiments.

Interestingly, the organism-level isotope effects reported for denitrification (Table 1) were often similar to the measured intrinsic isotope effect of nitrate reductases (15–30‰, with the caveat that these are for eukaryotic assimilatory reductases), indicating that the ratio of uptake to efflux among denitrifiers can approach unity ( $r_3 \approx r_4$ ). Unicellular algae appear more conservative in this regard, as isotope effects during nitrate assimilation are generally low (at a canonical 5‰), with some excursions toward the mid-teens (Waser et al. 1988a; Needoba et al. 2003; Granger et al. 2004). This may relate to the functional differences of nitrate between the two groups and/or to nitrate's relative availability in their respective environments. Cells that assimilate nitrate likely expend substantial energy to mitigate its loss, given a steep concentration gradient between the cells' interior and the environment (Dortch et al. 1984). In contrast, denitrifiers may forego the energetic expense of minimizing nitrate leakage in environments in which nitrate is abundant, and the effort to acquire nitrate at very low concentrations may be minimal given the relatively high demand for nitrate associated with anaerobic respiration.

*Variations in the magnitude of the N and O isotope effects*—Differences in the growth environment that led to substantial variations in the amplitude of the isotope effects among respiratory denitrifier cultures are elusive, given that conditions were intended to be similar among experiments. Unexplained variability in N isotope effects has been reported in previous studies of denitrifiers in batch culture (Wellman et al. 1968; Delwiche and Steyn 1970); however, few studies point to sensitivities of the isotope effect to environmental factors (Mariotti et al. 1981).

The potential physiological mechanisms underlying the variability in isotope effect amplitudes include both the enzyme-level isotope effect of NAR and the degree to which this enzymatic isotope effect is expressed externally. First, the intrinsic isotope effects of respective NAR enzymes could differ among strains, although we generally identified no obvious patterns in isotope effect amplitude specific to respective strains. Rather, the most prominent amplitude differences were observed within strains. Second, a sensitivity of the intrinsic isotope effect of NAR to the cellular

environment could underlie differences in the amplitude of the isotope effects. Bryan et al. (1983) observed that the N isotope effect intrinsic to the respiratory nitrite reductase *in vitro* is sensitive to both the concentration of nitrite as well as to the concentration of reductant provided to the enzyme. In direct analogy, the isotope effect of NAR could be sensitive to variations in the proximate concentrations of both internal nitrate and reductant, though this cannot be established from our observations. Finally, the process that likely imparts the largest variations in the amplitude of the organism-level isotope effects is the ratio of cellular nitrate uptake to efflux (Shearer et al. 1991; Needoba et al. 2004), although environmental factors that could have elicited physiological responses affecting this ratio in our cultures are not readily apparent.

Regardless of the specific mechanism of variation, one has to ask which overarching condition was most likely to vary in culture. The initial nitrate concentration was considerably different between cultures, yet this should have little direct influence on the isotope effect. However, it could bear indirectly on the isotope effect, to the extent that initial nitrate determined the concentration of nitrite intermediate that accumulated in some cultures. For instance, in one *Ochrobactrum* sp. culture (Table 2, experiment 33A), the initial nitrate concentration was upwards of 2 mmol L<sup>-1</sup>. While this strain generally accumulated no nitrite intermediate, this particular culture showed considerable nitrite accumulation, in proportion to nitrate consumption (data not shown). Nitrate uptake by denitrifiers is inhibited at high intracellular nitrite (Rowe et al. 1994), and nitrous acid can passively diffuse through the lipid bilayer, resulting in intracellular accumulation of nitrite. Thus, the relatively low <sup>15</sup>ε of 6‰ in the identified *Ochrobactrum* sp. culture could be the result of inhibition of nitrate uptake ( $r_3$ ) relative to the rate of nitrate reduction ( $r_5$ ), resulting in lowered proportional efflux of internal nitrate ( $r_4$  vs.  $r_3$ ).

Oxygen is one variable over which we had limited control during culturing. The cultures were grown in opaque, gas-impermeable bags, which do not preclude the presence of small air pockets or perhaps even small air leaks at the seams. Differences in the isotope effects among cultures could be telling of a sensitivity of efflux and uptake to oxygen concentrations in respective cultures. In denitrifiers, the expression of both the nitrate transporter and NAR is regulated by ambient oxygen. However, the activity of the transporter is reversibly inhibited by oxygen, while that of NAR is insensitive to the presence of oxygen (Alefounder and Ferguson 1980). Thus, as with the effect of nitrite accumulation, this differential regulation of the respective activities of NAR and nitrate transport could feasibly lead to variations in propagation of the internal enzymatic isotope effect, by causing variation in the relative proportion uptake and efflux ( $r_3$  vs.  $r_4$ ) among cultures.

Obviously, these suggestions for the origin of the differences in isotope effect amplitudes are highly speculative. The identification of environmental and physiological controls on the magnitude of the isotope effect during denitrification remains an important area for future work.

*Nitrate reduction by the periplasmic NAP*—The difference in the <sup>18</sup>ε: <sup>15</sup>ε that distinguishes *R. sphaeroides* from the other experimental strains also stands as an important result in this study. Nitrate reduction by *R. sphaeroides* in our cultures was ostensibly restricted to NAP activity. Since NAP is located in the periplasm of the cells, N and O isotope effects in external nitrate are presumably not affected by the relative rates of cellular nitrate uptake and efflux (Fig. 6). Therefore, the isotope effects measured for *R. sphaeroides* should approximate those intrinsic to NAP activity. Compared to some of the respiratory strains, both nitrate N and O isotope effects for *R. sphaeroides* were less variable, roughly ~15‰ and ~9‰, respectively, but they were not invariant. The observed variations may reflect physiological differences among cultures. For instance, the magnitude of N and O isotope effects among cultures could be telling of a sensitivity of the intrinsic isotope effects of NAP to the rate of supply of substrate or reductant (Bryan et al. 1983). In addition, as observed for respiratory denitrification, both the N and O isotope effects in one experiment with *R. sphaeroides* showed a distinct decrease below 30 μmol L<sup>-1</sup> nitrate, consistent with the Michaelis–Menten half-saturation constant ( $K_M$ ) of NAP in *R. sphaeroides*, estimated at 32 μmol L<sup>-1</sup> (Bursakov et al. 1997); this could indicate a dampening of the N and O isotope effects of NAP imposed by a sensitivity of the isotope effect of NAP to proximate nitrate and/or reductant (Bryan et al. 1983). However, because this trend is driven by a single sample, it should be taken as tentative.

Thus far, the potential impact of nitrate dissimilation by NAP during growth of the respiratory denitrifiers has not been considered. Unlike NAR, NAP does not provide a proton motive force to generate adenosine triphosphate and consequently is not involved in energy conservation during growth. While its expression is generally believed to be maximal during aerobic growth on very reduced substrate (Ellington et al. 2002), NAP expression is also reportedly required for transition to anaerobeosis in a *Pseudomonas* sp. strain (Bedzyk et al. 1999) and has further been documented during anaerobic growth in some denitrifying strains (Bedzyk et al. 1999). We cannot distinguish whether nitrate reduction by NAP occurred during aerobic and anaerobic growth of the respiratory denitrifiers, but we still must consider that it potentially dissimilated some nitrate concomitantly with NAR. Given the  $\Delta\delta^{18}\text{O}:\Delta\delta^{15}\text{N}$  of ~1 for eukaryotic nitrate assimilation, one might assume that the true value for NAR is also 1 and that nitrate reduction by NAP could lower the  $\Delta\delta^{18}\text{O}:\Delta\delta^{15}\text{N}$  toward a value of 0.6. Arguably, the <sup>18</sup>ε: <sup>15</sup>ε observed among the respiratory denitrifiers is not 1 but rather ≤1, as it ranged between 0.86 to 1.02. Thus, the apparent departure of the  $\Delta\delta^{18}\text{O}:\Delta\delta^{15}\text{N}$  coupling of *P. stutzeri* from other respiratory denitrifiers (~0.89 vs. ~0.97) could be indicative of NAP expression during anaerobic growth of this strain. In any case, differential expression of NAR vs. NAP by denitrifiers needs to be examined more closely to determine whether NAP modulates nitrate N and O isotope effects expressed by respiratory denitrifiers.

It is remarkable that phylogenetic relatedness among reductase types is not prognostic of the intrinsic isotope

effect. As noted earlier, the constant coupling of  $\sim 1$  between the  $\Delta\delta^{18}\text{O}:\Delta\delta^{15}\text{N}$  isotopic enrichment deduced here for NAR is indistinguishable from that deduced for the eukaryotic nitrate reductase, eukNR, during nitrate assimilation among strains of unicellular eukaryotic phytoplankton (Granger et al. 2004). This similarity is unexpected, because the assimilatory eukNR and the dissimilatory NAR are sufficiently different from each other to warrant classification in separate families of molybdo-enzymes, namely the sulfite-oxidase family for the eukaryotic assimilatory eukNR and the dimethyl sulfoxide (DMSO)-reductase family for NAR (Moreno-Vivian et al. 1999). The similar coupling may reflect a convergence of the two enzyme types upon the most kinetically favorable catalysis for nitrate reduction.

The observation of a distinct  $\Delta\delta^{18}\text{O}:\Delta\delta^{15}\text{N}$  imparted by NAP relative to NAR is significant because it reveals that different types of nitrate reductases can have different intrinsic isotope effects, in spite of their ultimately catalyzing the same chemical reaction. The difference in NAP's intrinsic N and O isotope effects from those of NAR is surprising in that NAP belongs to the DMSO-reductase family of molybdo-enzymes and is thus more closely related to NAR than NAR is to eukNR. The respective functional roles of NAP and NAR (and eukNR) may underlie this difference. While NAR is involved in energy conservation, NAP mitigates energy dissipation. Arguably, all NAP enzymes ultimately may not have this same  $\Delta\delta^{18}\text{O}:\Delta\delta^{15}\text{N}$ , yet the catalytic subunit of NAP is highly conserved among bacterial clades (Moreno-Vivian et al. 1999). More broadly, the magnitude of the enzymatic isotope effects and the sensitivity of these to the cellular environment may provide important insight into the catalytic mechanisms of respective nitrate reductases.

*The freshwater conundrum*—The  $^{18}\text{E}:\text{E}^{15}$  of  $\sim 1$  associated with respiratory denitrification provides an important constraint for interpretation of coupled measurements of nitrate N and O isotopes, and it seems consistent with other findings from cultures (Granger et al. 2004) and from field studies in the ocean (Sigman et al. 2005). However, the apparent difference in  $^{18}\text{E}:\text{E}^{15}$  documented in marine vs. freshwater systems, with  $\sim 1$  in the former and  $\sim 0.6$  in the latter (Lehmann et al. 2003, and references therein), remains a puzzle. In our culture experiments, differences in medium salinity did not seem to change the observed coupling between N and O isotopes of the denitrifiers and thus do not serve as an explanation. The same can be said of pH, which was roughly 0.5 units higher in saline medium (8.1 vs. 7.6), yet which caused no change in the  $\Delta\delta^{18}\text{O}:\Delta\delta^{15}\text{N}$  of denitrifiers. Moreover, we observed no distinction in heavy isotope enrichment of nitrate between marine vs. freshwater bacterial isolates. The  $^{18}\text{E}:\text{E}^{15}$  of  $\sim 0.6$  reported for freshwater systems has hitherto been attributed to isotopic fractionation imparted by denitrification. This hypothesis is reportedly supported by *in vitro* measurements of nitrate N and O isotope fractionation by a dissimilatory nitrate reductase showing coupled fractionation ( $^{18}\text{E}:\text{E}^{15}$ ) of 0.6 (Olleros [1983], as cited by Amberger and Schmidt [1987]). However, the aforemen-

tioned work relied upon an off-line pyrolysis-based oxygen isotope method, which can dramatically underestimate nitrate  $\delta^{18}\text{O}$  variations (as a result of O isotope exchange with the quartz pyrolysis tube [Casciotti et al. 2002; Revesz and Böhlke 2002]). Moreover, it was not determined whether the measured nitrate N and O isotope effects were those intrinsic to NAP or NAR.

Based on our findings, the  $^{18}\text{E}:\text{E}^{15}$  ratio observed in freshwaters is conspicuously similar to that observed here for the photo-heterotroph *R. sphaeroides* and, hence, is close to the  $^{18}\text{E}:\text{E}^{15}$  ratio of NAP. But NAP is not a respiratory enzyme per se and is therefore not likely to be of great significance with respect to nitrate loss in terrestrial systems at low oxygen tension. Consequently, the discrepancy between the  $\Delta\delta^{18}\text{O}:\Delta\delta^{15}\text{N}$  observed here for denitrifiers possessing NAR (close to 1.0) and that of 0.6 observed in freshwater is not reconcilable at this point. Our results have the exciting implication that this conundrum may portend of unidentified, yet fundamental, differences between the terrestrial and oceanic N cycles.

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Received: 25 July 2007

Accepted: 24 May 2008

Amended: 11 June 2008