8-17-2015

Enzyme Level N and O Isotope Effects of Assimilatory and Dissimilatory Nitrate Reduction

Lija Treibergs
University of Connecticut - Avery Point, lija440@gmail.com

Enzyme Level N and O Isotope Effects of Assimilatory and Dissimilatory Nitrate Reduction

Lija A. Treibergs
B.A., Princeton University, 2012

A Thesis
Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science
At the University of Connecticut
2015

APPROVAL PAGE

Masters of Science Thesis

Enzyme Level N and O Isotope Effects of Assimilatory and Dissimilatory Nitrate Reduction

Presented by

Lija A. Treibergs, B.A.

Major Advisor_____
Julie Granger

Associate Advisor _____
Craig R. Tobias

Associate Advisor_____
Pieter T. Visscher

University of Connecticut

2015

Acknowledgements:

I would like to thank my advisor, Dr. Julie Granger, as well as the rest of my advising committee, Drs. Craig Tobias and Pieter Visscher for their guidance in this work. I would also like to thank the Granger and Tobias labs, specifically Richard Dabundo, Sydney Twarz and David Cady, for their assistance in lab work and sample analysis. Finally, I would like to thank everyone in the Department of Marine Sciences at the University of Connecticut and my family for their support in my pursuit of this endeavor.

Abstract:

Nitrate N and O isotope distributions in the environment can be used to elucidate salient biogeochemical N transformations. In order to do so, it is necessary to know the isotopic imprints characteristic of respective N transformations, and to understand the underlying mechanisms that determine these patterns. In order to provide mechanistic constraints on the isotopic imprints associated with nitrate consuming processes, we measured the enzymatic N and O isotope effects ($^{15}\varepsilon$ and $^{18}\varepsilon$) imparted on nitrate by three types of nitrate reductase enzymes, including (a) a prokaryotic respiratory nitrate reductase, *Nar*, from the heterotrophic denitrifier *Paracoccus denitrificans*, (b) a prokaryotic periplasmic nitrate reductase, *Nap*, from the photoheterotroph *Rhodobacter sphaeroides*, and (c) two commercially purified extracts of eukaryotic assimilatory nitrate reductases (EukNR) from *Pichia angusta* and from *Arabidopsis thaliana*. Enzymatic *Nar* assays fuelled with the artificial electron donors methyl and benzyl viologen yielded identical N and O isotope effects ($\Delta\delta^{18}O{:}\Delta\delta^{15}N \approx 1$) of ~27‰, regardless of the initial nitrate concentration (200 μM *vs.* 1000 μM) or assay temperature (20˚C *vs.* 4˚C). Enzymatic assays with EukNR fuelled by methyl viologen yielded strikingly identical results to *Nar*, namely a $\Delta\delta^{18}O{:}\Delta\delta^{15}N \approx 1$ and isotope effect magnitudes of ~27‰. *Nar* assays fuelled with the physiological reductant hydroquinone also yielded a consistent $\Delta\delta^{18}O{:}\Delta\delta^{15}N \approx 1$, but showed more variable isotope effect amplitudes, from 22.9 ± 1.5‰ to 33.0 ± 4.3‰. This suggests that isotope effect amplitudes may be sensitive to the rate of internal electron transfer to the enzyme's catalytic site. *Nap* assays showed unique fractionation patterns, including a $\Delta\delta^{18}O{:}\Delta\delta^{15}N \approx 0.5$, N isotope effect of ~38‰, and O isotope effect of ~19‰, which portends a different catalytic mechanism than that of the closely related *Nar* and distantly related EukNR enzyme types. These results confirm that dominant nitrate consuming processes in the environment fractionate with a $\Delta\delta^{18}O{:}\Delta\delta^{15}N \approx 1$, providing a reliable benchmark from which to identify their specific signature from environmental isotope distributions. The distinctive isotopic signature of the auxiliary *Nap* enzyme is of interest with respect to deciphering catalytic mechanisms, but is unlikely to account for imprints on nitrate in the environment given the auxiliary role of *Nap* in bacterial physiology.

Introduction:

Nitrogen (N) is an essential nutrient for life, whose availability has substantial influence on the productivity of terrestrial and marine ecosystems (Falkowski 1997; Gruber 2008; Gruber and Galloway 2008). It is thus important to understand the sources and sinks of bioavailable nitrogen on global and regional scales. To this end, the naturally occurring stable N and O isotope ratios of nitrate ($^{15}N/^{14}N$ and $^{18}O/^{16}O$, respectively) can be used as indicators of sources, sinks, and transformation processes among N pools (*e.g.*, Casciotti et al. 2002; Sigman et al. 1997; Sigman et al. 2005). The isotopic composition of nitrate registers the isotopic imprints of its source(s) as well as those imparted by of the transformations to which it was subject, thus integrating the spatial and temporal variability inherent to N transformations in the environment, which is difficult to capture otherwise. Measured in tandem, the coupled N and O isotope ratios of nitrate also provide complementary signatures of co-occurring N transformations that could not be disentangled from measurements of nitrate N isotope ratios alone (e.g., Sigman et al. review 2009).

The two major biological nitrate consumption pathways in the N cycle are nitrate assimilation and denitrification, the latter of which constitutes microbially-mediated respiratory reduction of nitrate to N_2 gas. Both of these reactions impart N and O isotopic enrichment to the unconsumed nitrate pool. During assimilation and denitrification, nitrate containing the light isotopes, ^{14}N and ^{16}O, reacts faster than that with the heavy isotopes, leading to an progressive enrichment of both ^{15}N and ^{18}O of the remaining nitrate pool as nitrate is consumed (Wada and Hattori 1978; Granger et al. 2004; Granger et al. 2008). The degree to which isotopic discrimination occurs is quantified by the kinetic isotope effect, $\varepsilon = ({}^{light}k/{}^{heavy}k - 1) \times 1000$,

expressed in per mille (‰), where the $^{light}k$ and $^{heavy}k$ are the respective reaction rate coefficients for the heavy and the light isotope bearing molecules.

Culture studies of nitrate uptake by marine phytoplankton and by denitrifying bacteria have elucidated that the magnitude of the organism-level isotope effect (ε_{org}) imparted on nitrate during both nitrate assimilation and nitrate respiration is determined by analogous processes, and is largely dependent on the fate of nitrate after its active uptake into the cell (*see* Figure 1) (Shearer 1991; Granger et al. 2004, 2008, 2010; Needoba et al. 2004). Of the nitrate actively transported into the cell, a fraction is irreversibly reduced to nitrite by the respective assimilatory or respiratory nitrate reductase enzymes, whereas a portion of the internal nitrate escapes enzymatic reduction and passively effluxes back into the environment, given a favorable electrochemical gradient. Isotope discrimination of the heavy isotopologues of nitrate occurs internally during bond-breakage at enzyme site, and not during transport (Karsh et al. 2014; Granger et al. 2008; Granger et al. 2010; Kritee et al. 2012). The magnitude of ε_{org} recorded in the external medium thus records that imposed by the enzymatic isotope effect, but varies as a function of the relative fraction of nitrate effluxed out of the cell, where $\varepsilon_{org} = f * (\varepsilon_{enzyme})$ and f is the ratio of nitrate efflux to uptake (Shearer et al. 1991; Francois et al. 1993;Needoba et al. 2004; Karsh et al. 2014). For assimilatory nitrate reduction mediated by eukaryotic nitrate reductase (EukNR), the N isotope effect ($^{15}\varepsilon_{EukNR}$) of nitrate reductase purified from the fungus *Aspergillus* sp., chosen due to its commercial availability as a purified enzyme, and from cell suspensions of marine diatom *Thalassiosira weissflogii*, were recently shown to be ~27‰ *in vitro* (Karsh et al. 2012), which is coherently higher than the upper end of the assimilation isotope effect as observed in cultures, which ranges from 0 to 20‰ (Wada and Hattori 1978; Montoya and McCarthy 1995; Waser et al. 1998a; Needoba et al. 2003; Granger et al. 2004), and from that

observed at the surface ocean, which ranges from 5 to 10‰ (Wada 1980; Wu et al. 1997; Waser et al. 1998; Sigman et al. 1999; Altabet 2001; DiFiore et al. 2006). N isotope effects in cultures of denitrifiers and those attributed to denitrification in the environment cover a broader range than observed for nitrate assimilation, from 2 to 30 ‰ (Wellman et al. 1968; Wada et al. 1975; Barford et al. 1999; Brandes et al. 1998; Voss et al. 2001; Granger et al. 2008). The enzymatic isotope effect associated with nitrate reduction by the respiratory *Nar* nitrate reductase is not well constrained. Because the enzymatic isotope effect would not be influenced by the relative fraction of nitrate effluxed out of the cell as described above, we anticipate it to be greater than the denitrification isotope effects observed in cultures and in the environment. It would thus set an upper boundary for the isotope effect of denitrification, which is on the order of 25 to 30‰ (Wellman et al. 1968; Barford et al. 1999; Granger et al. 2008).

Understanding the magnitude of the N isotope effects of denitrification and the factors that control it is of importance due to the utility of N isotopes in constraining source and sink estimates of fixed N to the global ocean (Brandes and Devol 2002). Global estimates of N_2-fixation and of water-column and benthic denitrification, which are derived from extrapolations of field rate measures or from geochemical estimates, vary widely. The oceanic N budget can thus be construed as being relatively balanced on ocean-circulation time scales (Gruber and Sarmiento 1997; Gruber 2008) or grossly out of balance, losing nitrate at a faster rate than is being produced (Altabet and Curry 1989; Brandes and Devol 2002; Canfield et al. 2010; Codispoti 1995; Deutsch et al. 2007). In this respect, N isotope ratios and isotope effects of pertinent N transformations provide an additional conserved metric from which to construct a mass balance of nitrogen sources and sink terms to the global ocean. In these exercises, the isotope effect associated with water column denitrification, $^{15}\varepsilon_{denit}$, is generally presumed to be

on the order of 25‰ (Brandes and Devol 2002), even though $^{15}\varepsilon_{denit}$ has been found to vary from 2 and 30‰ in both the water column and culture studies (Brandes et al. 1998; Voss et al. 2001; Granger et al. 2008). Estimates of the magnitude of the benthic denitrification term derived therein, which assume the isotope effect associated with benthic denitrification is near 0‰, result in bulk denitrification (water column and benthic) that is over 2 x greater than estimates of N_2-fixation rates, thus diagnosing a massive and improbable imbalance in the modern oceanic N budget. Recent investigations that looked into the variability in $^{15}\varepsilon_{denit}$ with cell specific nitrate reduction rates in cultures grown under various conditions relevant to the ocean have called into question the validity of setting the $^{15}\varepsilon_{denit}$ of water column denitrification at 25‰, as under many of the simulated 'oceanic' growth conditions, where cell specific nitrate reduction rate was lower than for culture studies under ideal growth conditions, the isotope effect was driven lower than the canonical 25‰ (Kritee et al. 2012). Based on the results of their experiments, the authors suggest a value of 10-15‰ may be more appropriate for N budget mass balance exercises, and a lower value would mitigate the discrepancy between the magnitudes of the source and sink fluxes to the fixed nitrogen pool. A balanced N-budget is attractive; however, given the importance of the magnitude of $^{15}\varepsilon_{denit}$ of denitrification, it is worth trying to further constrain this value for its use in models and to understand what determines its expression in the environment.

Characterizing the coupling between nitrate N and O isotope effects associated with nitrate consumption and identifying the mechanisms that influence it are also important, as the nitrate N-to-O relationship provides a basis from which to identify when nitrate consumption by assimilation and denitrification is occurring in tandem with nitrate production from nitrification in the environment. Both denitrification and nitrogen assimilation have been observed to have N

and O isotope effects that co-vary linearly with a ratio near 1 ($^{18}\varepsilon$:$^{15}\varepsilon \cong 1$) in culture studies (Granger et al. 2004; Granger et al. 2008; Kritee et al. 2012; Wunderlich et al. 2013) as well as in the marine environment (*e.g.*, Casciotti et al. 2002; Sigman et al. 2003). It has been shown that this holds true at the enzyme level for nitrate assimilation: pure extracts of assimilatory eukaryotic nitrate reductase (EukNR) from the fungus *Aspergillus* sp. and for a marine diatom fractionate N and O isotopes with a ratio ≅ 1:1 *in vitro* (Karsh et al. 2012), replicating the O-to-N observed at the organism level, and confirming unequivocally that enzymatic reduction is the fractionating step during assimilation. This peculiar signature constitutes a benchmark in studies using coupled N and O isotope measurements; deviations from the $^{18}\varepsilon$:$^{15}\varepsilon$ ratio of 1 in environmental samples provide an indication of co-occurring N transformations, where multiple processes are adding to or removing nitrate from a pool simultaneously. In particular, ratios above 1 have been found in the oxygen deficient zone of the Eastern Tropical North Pacific (ETNP) near the shelf break off of Baja California and have been tentatively attributed to the ammonification and subsequent nitrification of newly fixed organic material low in $\delta^{15}N$ or the re-oxidation of NO_2^- previously reduced from NO_3^- (Sigman et al. 2005; Casciotti and McIlvin 2007). In the latter scenario, newly reduced NO_2^- has a $\delta^{15}N$ lower than that of its source NO_3^-- due to the fractionation associated with NO_3^- reduction, and upon re-oxidation it produces NO_3^- low in $\delta^{15}N$, but the $\delta^{18}O$ of the produced NO_3^- has the isotopic imprint near that of ambient seawater at ~1‰ (Sigman et al. 2009; Buchwald and Casciotti 2010). This explanation has also been used to explain deviations from 1:1 signal of denitrification seen in the Peruvian ODZ, where NO_2^- re-oxidation may provide an equally or more important sink for NO_2^- as denitrification (Casciotti et al. 2013). In the surface ocean, nitrification occurring subsequently with nitrate assimilation can similarly lead to an $^{18}\varepsilon$:$^{15}\varepsilon > 1$ depending on the fate of the NH_4^+

pool; NH_4^+ will be utilized as an N source for assimilation in addition to being nitrified. The partitioning of NH_4^+ between assimilation and nitrification, as well as the relative amplitudes of their respective isotope effects, modulate the $\delta^{15}N$ of the newly nitrified NO_3^- and thus the amplitude of the deviation from the 1:1 signal imposed by nitrate assimilation (Wankel et al. 2007; Di Fiore et al. 2009; Smart et al. 2015). Due this complexity, the magnitudes of specific processes are difficult to parse out from the isotopic signals when co-occurring transformations are at work.

In freshwater systems, however, denitrification is associated with coincident increase in O and N isotopes signal whose $^{18}\varepsilon:^{15}\varepsilon$ is between 0.5 and 0.7 (Lehman et al. 2003; Amberger and Schmidt 1987; Knöller et al. 2011), thus below the 1:1 observed for cultures of denitrifying bacteria and in marine systems. This prevalent signal has traditionally been interpreted as the organism-level isotope effect for denitrification (Amberger and Schmidt 1987), yet this premise is clearly contradictory to observations from culture work, which demonstrates a consistent $^{18}\varepsilon:^{15}\varepsilon$ of 1 among various strains of denitrifiers (Granger et al. 2008; , Kritee et al. 2012; Wunderlich et al. 2013) Some observations from culture work, however, have led to speculations that O-to-N is malleable, ranging from as low as 0.3 up to 1.0, depending on culture conditions (Knöller et al. 2011), thus providing a potential explication for the lower $^{18}\varepsilon:^{15}\varepsilon$ observed in freshwater systems.

In order to explain the discrepancy between the canonical 1:1 of marine systems and the <1 observed in freshwater systems, some workers have also suggested that denitrification in freshwater systems is catalyzed by an alternate dissimilatory nitrate reductase enzyme, the periplasmic *Nap* nitrate reductase (Wenk et al. 2014, Frey et al. 2014), rather than the respiratory *Nar* nitrate reductase. Indeed, many denitrifying organisms possess an auxiliary *Nap* nitrate

reductase enzyme, which is located in the bacterial periplasm. *Rhodobacter sphaeroides,* a photo-heterotrophic bacterium possessing only *Nap,* has been shown to fractionate the O and N isotopes of nitrate at a ratio below 1 in culture (~0.6,) suggesting that *Nap* fractionates oxygen and nitrogen isotopes differently from the other nitrate reductases (Granger et al, 2008). Similarly, Frey et al. (2014) recently measured a $^{18}\varepsilon:^{15}\varepsilon$ of ~0.5 during the growth of an autotrophic sulfide-oxidizing Epsilon-proteobacteria, *Sulfurimonas gotlandica*, which also possesses *Nap* as its sole nitrate reductase enzyme. For organisms that possess both *Nap* and *Nar*, the organism level isotope effect, ε_{org}, should be determined by the ratio of efflux to uptake (*f*, as described above) and the combined enzymatic isotope effect of *Nar* and *Nap*. With this assumption, we might expect the $\delta^{18}O:\delta^{15}N$ during nitrate respiration of organisms possessing both *Nar* and *Nap* to be below 1:1 depending on the relative activity of *Nap* to *Nar*. This perhaps could come into play in freshwater systems, where redox conditions could favor the expression and activity of *Nap* and thus lower the observed $^{18}\varepsilon:^{15}\varepsilon$.

In order to provide additional constraints on the $^{18}\varepsilon:^{15}\varepsilon$ associated with nitrate consumption and to further establish the O-to-N coupling as robust benchmark to interpret nitrate isotope distributions in the environment, we measured the nitrate N and O isotope fractionation imposed on nitrate by various nitrate reductase enzymes in cell homogenates or in purified enzymatic extracts. Our results confirm trends observed previously for other eukaryotic assimilatory nitrate reductases, and provide novel observations of the N and O isotope effect imparted by the respective prokaryotic *Nar* and *Nap* enzymes.

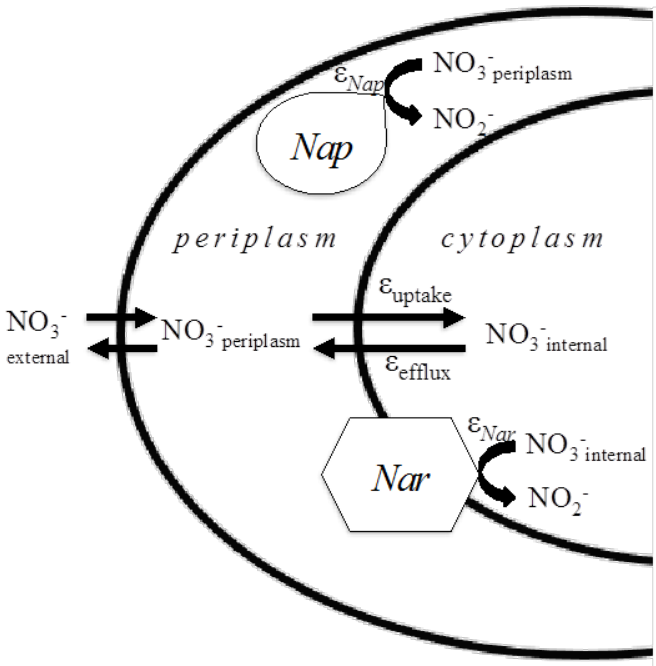

Figure 1: The organism level isotope effect , ε_{org}, which is the isotope effect expressed on nitrate in the environment, is dependent on a variety of processes within the cell, each with their own isotope effect, and these processes' relative importance to each other. For denitrifiers, this is dependent on uptake of nitrate into the cell, efflux of unconsumed nitrate out of the cell, and the first irreversible step of denitrification; the enzymatic reduction of nitrate to nitrite, such that $\varepsilon_{org} = f(\varepsilon_{enzyme})$ and f is the ratio of efflux to uptake. Some denitrifiers posses two nitrate reducing enzymes, *Nar* and *Nap,* and each could have an independent isotope effect contributing to ε_{enzyme}.

Materials and Methods:

Sources of Nitrate Reductase

Enzymatic assays were conducted on (a) cell homogenates from the denitrifying bacterial strain *Paracoccus denitrificans* (American Type Culture Collection [ATCC] 19367) cultured under anaerobic vs. aerobic conditions, on (b) cell homogenates from the photo-heterotrophic bacterial strain *Rhodobacter sphaeroides* (Deutsche Sammlung von Mikroorganismen [DSM] 158) cultured aerobically, and on (c) purified extracts of recombinant eukaryotic assimilatory nitrate reductases (EukNR) from the flowering plant *Arabidopsis thaliana (*AtNaR*:* E.C. 1.7.1.1) and from the yeast *Pichia angusta* (YNaR1: E.C. 1.7.1.2), both purchased from NECi

(nitrate.com).

(a) *Paracoccus denitrificans* cell concentrate preparations

Paracoccus denitrificans culture medium contained 30 g L^{-1} Fisher Scientific Bactro™ tryptic soy broth supplemented with 300 μmol L^{-1} KNO_3, 1 mmol L^{-1} $NaNH_4$, and 100 μmol L^{-1} K_2HPO_4. NH_4^+ was added in excess to inhibit the expression of assimilatory nitrate reductase, *Nas*, and ensure that all NO_3^- was instead being reduced by *Nar* or *Nap* (Bender and Friedrich 1990). Media were then sterilized by autoclaving for 1 hour. A large culture was initiated in an acid washed 2 L Erlenmeyer flask and grown at room temperature while continuously purged with lab air. After 3 days, when cell density was maximal, the flask was sealed to cut off the oxygen supply and to allow for the inception of denitrification. The culture was thus left for 14 hours, after which it was tested for presence of NO_3^- and NO_2^- to confirm complete removal. Cells were harvested by centrifugation for 20 minutes at 12,000 g. The cell pellet was resuspended in a 100 µM potassium phosphate buffer solution [pH 7.9] containing Thermo Scientific Halt™ Protease Inhibitor Cocktail and 100 µmol L^{-1} ethylenediaminetetraacetic acid (EDTA,) immediately flash frozen in liquid nitrogen, and transferred to a -80º freezer for long term storage.

An additional culture of *P. denitrificans* was grown under aerobic conditions to favor the expression of *Nap* and the suppression of *Nar*. Media specifications and growth conditions were the same as above. The culture, however, was purged with air continuously until harvest, to inhibit the expression of Nar but not *Nap* (Korner and Zumft, 1989). Prior to harvest, the culture was kept on ice during transport to the centrifuge in hopes to minimize any possible expression of *Nar* when cells were not being purged with air. Cell pellets were resuspended in buffered solution and flash frozen as above.

(b) *Rhodobacter sphaeroides* cell concentrate preparations

In order to provide cellular extracts for *Nap* reductase assays, the photo-heterotrophic bacterial strain *Rhodobacter sphaeroides* was grown in a modified RCV medium (Weaver et al. 1975) containing 4 g L^{-1} Bactro™ tryptic soy broth amended with 300 μM KNO_3, 4 g L^{-1} $MgSO_4$, 1.5 g L^{-1} $CaCl_2$, 40 mL L^{-1} 1% wt/vol EDTA and 0.05 g L^{-1} $NaNH_4$. NH_4^+ was added in excess to inhibit the expression of the prokaryotic assimilatory nitrate reductase *Nas* and ensure all NO_3^- was being reduced by *Nap*. After autoclaving for 1 hr, the medium was further amended with 0.2 µM filter sterilized, 0.2 M phosphate buffer [pH 6.8], 1 mL L^{-1} Teknova T1001 trace metal mix, and 1 mL of *f/2* vitamins (Guillard 1975). A large batch culture was initiated in an acid washed 2 L Erlenmeyer flask and grown at room temperature while continuously purged with lab air. Cells were harvested by centrifugation, and resuspended in buffered solution as above.

(c) Commercial stocks of purified eukaryotic nitrate reductases (EukNR)

Freeze dried commercially prepared purified eukaryotic nitrate reductase (EukNR) enzyme preparations from *Arabidopsis thaliana* and *Pichia angusta* were purchased from NECi (nitrate.com) and reconstituted in 1 mL of the accompanying assay buffer solution (25 mmol L^{-1} KH_2PO_4 [pH 7.5], 25% glycerol vol/vol, 25 µmol L^{-1} EDTA) to an activity of 1 unit, defined as sufficient enzyme activity to reduce 1 µmol L^{-1} min^{-1} at 25º C.

Enzymatic assay preparations:

Initial *Nar* assays (1 and 2) used the anaerobically-grown *P. denitrificans* cell suspension directly from the frozen stock with no additional preparation. In all subsequent cell suspension assays (*P. denitrificans* and *R. sphaeroides*), the frozen stock of cell suspension was thawed in ice water to minimize enzyme degradation, and working fractions were supplemented with 1% *v/v* Triton-X 100 and subjected to 2 freeze-thaw cycles in liquid nitrogen to further promote membrane breakdown and protein solubilization.

P. denitrificans assays were conducted either at room temperature (~20º C) or in a cold room maintained at 4º C to assess potential temperature effects on the enzymatic isotope effect of dissimilatory nitrate reductases. Prior to performing these experiments, all reagents were pre-chilled to 4º C in the cold room. All *R. sphaeroides* and EukNR assays were conducted at room temperature.

Assays contained 0.5 or 1 mL of cell suspension or of commercially purified EukNR buffered solution, 1 mL of 200 μmol L^{-1} reducing agent – either membrane-permeant benzyl viologen dichloride [Sigma-Aldrich, CAS: 1102-19-8], methyl viologen dichloride hydrate [Sigma-Aldrich, CAS: 75365-73-0], or hydroquinone (for dissimilatory reductases only; [MP Organics]) – 0.2 or 1 mL 10 mmol L^{-1} KNO_3 to a final concentration of 200 or 1000 μmol L^{-1}, and the remaining volume of 100 mmol L^{-1} phosphate buffer [pH 7.9] containing 100 μmol L^{-1} to a final assay volume of 10 mL. After removing an (initial) aliquot of 1 mL (sample) for quantitation of [NO_3^-] and [NO_2^-], the denitrification reaction was commenced by the addition of 1 mL of 57 mmol L^{-1} sodium dithionite in 29 mmol L^{-1} sodium bicarbonate, which reduces the electron donor. Initial [NO_3^-] and [NO_2^-] values are corrected for this dilution. Sequential 1 mL samples were drawn approximately every 90 seconds during room temperature assays and every 3 minutes during assays conducted at 4°C. Samples were mixed vigorously on a vortex mixer for

30 s immediately upon collection to halt the reaction through oxidation of the methyl or benzyl viologen or hydroquinone. In selected assays, additional ~ 50 µL samples were also drawn throughout the assay reactions for determination of $[NO_2^-]$ and were measured immediately. In order to ensure complete cessation of enzyme activity, samples placed in an 80º C water bath for 2 to 10 minutes. NO_2^- was then removed from the samples *via* the addition of 55 µL 4% (wt/vol) sulfamic acid in 10% vol/vol HCl (Granger and Sigman 2009; Karsh et al. 2012). For two assays (see Supplement Materials SI), subsets of samples were also subject to an alternate NO_2^- removal method using ascorbic acid under He purging (Granger et al. 2006) to compare NO_2^- removal effectiveness at elevated $[NO_2^-]$ to $[NO_3^-]$ ratios. Following nitrite removal, samples were returned to neutral pH with the addition of concentrated NaOH and frozen for short-term storage.

Determination of $[NO_2^-]$ and $[NO_3^-]$

$[NO_2^-]$ was measured in the 50 µL samples by chemiluminescence detection on a NO_x analyzer (model T200 Teledyne Advanced Pollution Instrumentation) following reduction to nitric oxide (NO) in a heated iodine solution (Garside 1982). $[NO_3^-]$ was also determined by chemiluminescence detection on the NO_x analyzer following conversion to NO in a heated vanadium solution (Braman and Hendrix 1989). Nitrite had been previously removed from these samples, as vanadium reduces both nitrate and nitrite to NO.

Determination of nitrate $\delta^{15}N$ and $\delta^{18}O$

NO_3^- $\delta^{15}N$ and $\delta^{18}O$ were determined with the denitrifier method (Sigman et al. 2001; Casciotti et al. 2002), wherein denitrifying bacteria lacking terminal nitrous oxide reductase (*P. chlororaphis* f. sp. *aureofaciens* ATCC 1398) quantitatively convert sample NO_3^- to N_2O gas, which is then extracted, purified and analyzed through a modified Thermo-Scientific Gas Bench II and Delta V Advantage gas chromatograph isotope ratio mass spectrometer. Samples were

standardized through comparison to reference standards IAEA-N3, USGS-34, and USGS-32, which have $\delta^{15}N$ (*vs* air N_2) and $\delta^{18}O$ (*vs* V-SMOW) of 4.7‰ and 25.6‰, -1.8‰ and -27.9‰, and 180‰ and 25.6‰ respectively (Gonfiantini et al. 1995; Böhlke et al. 2003) after individually being referenced to pure N_2O injections from a common reference gas cylinder. Samples were also corrected for a bacterial 'blank' when present, defined as any N_2O produced by bacteria in the absence of sample injection.

The N and O isotope effects, $^{15}\varepsilon$ and $^{18}\varepsilon$, were derived from the slope of the linear fit of $[NO_3^-]$ *vs*. $\delta^{15}N$ or $\delta^{18}O$ according to the Rayleigh approximation for fractionation of a substrate in a closed system (Mariotti et al. 1981). Error on the slope – and thus on the isotope effect – was calculated using model II geometric mean regression analysis that factors error associated with individual measures on both the x- and y-coordinates (Sokal and Rohlf 1995) . Standard deviations for $\delta^{15}N$ and $\delta^{18}O$ were calculated from analytical replicates. Measurement errors for $[NO_3^-]$ were assigned a 3% of the reported $[NO_3^-]$, a representative estimate based on the mean precision of $[NO_3^-]$ measured from standards of known concentrations.

Results

$[NO_3^-]$ decreased with time in all nitrate reductase assays. In assays where $[NO_2^-]$ was measured concurrently, it accumulated to a concentration equivalent of the coincident NO_3^- drawdown. Dissimilatory *Nar* and *Nap* nitrate reductase assays with methyl viologen reacted faster than corresponding assays with hydroquinone, and assays at 4ºC also proceeded more slowly than corresponding assays at room temperature. NO_2^- removal with sulfamic acid was only effective to a point around 1:25 to 1:50 $[NO_3^-]$:$[NO_2^-]$, evidenced in part by a tendency for nitrate concentrations to plateau at low concentrations (around 20-40 μM) in association with

haphazard O vs. N isotope ratios. In two assays in which NO_2^- was removed with the ascorbic acid method revealed complete NO_3^- drawdown and coherently linear Rayleigh distillation at lower [NO_3^-]. Thus, estimates of isotope effects describe the linear portion of the Rayleigh linearization and other points were discarded.

For all assays with anaerobic and aerobic cell suspensions of *P. denitrificans*, the respective magnitudes of the change in $\delta^{15}N$ and $\delta^{18}O$ were roughly similar in all assays, regardless of cell growth conditions, of initial [NO_3^-], reductant type, or assay temperature (Table 1, Figure 2a). The ratios of $^{18}\varepsilon$ to $^{15}\varepsilon$, determined by the change in $\delta^{18}O$ ($\delta^{18}O$ *minus* $\delta^{18}O_{initial}$*) vs.* the change in $\delta^{15}N$ ($\delta^{15}N$ *minus* $\delta^{15}N_{initial}$), hereafter referred to as $\Delta\delta^{18}O$: $\Delta\delta^{15}N$, were thus consistently near 1. The magnitude of the $^{15}\varepsilon$ (and coupled $^{18}\varepsilon$) determined by Rayleigh linearization varied among *P. denitrificans* assays, between 6.6 and 33.0‰. The lowest $^{15}\varepsilon$ and $^{18}\varepsilon$, on the order of 6 to 9‰ were only observed for assays fuelled by benzyl viologen with unlysed cell suspensions. Assays with lysed cell suspensions fuelled by either benzyl viologen or methyl viologen yielded isotope effects ranging from 26.0 ± 1.9 to 28.9 ± 1.0 ‰ with an average of 27.7 ± 0.6 ‰. An analogous assay with methyl viologen conducted at 4˚C yielded an identical $^{15}\varepsilon$ of 27.6 ± 1.9‰.

Assays of lysed *P. denitrificans* homogenate fuelled by hydroquinone as the reductant yielded a broader $^{15}\varepsilon$ (and $^{18}\varepsilon$) range, from 22.9‰ to upwards of 33.0‰. The hydroquinone assays with 1 mmol L^{-1} [NO_3^-] conducted at room temperature differed among batches (*i.e.*, assay dates) ranging from 29.9 ± 3.3‰ to as high as 33.0 ±4.3‰ on one occasion *vs*. 26.5 ± 0.9 ‰ and 27.5 ± 1.0 ‰ at a second occasion. Hydroquinone assays at lower temperature yielded isotope effects between 26.1 ± 10.3 ‰ and 27.3 ± 1.4‰, indistinguishable from some

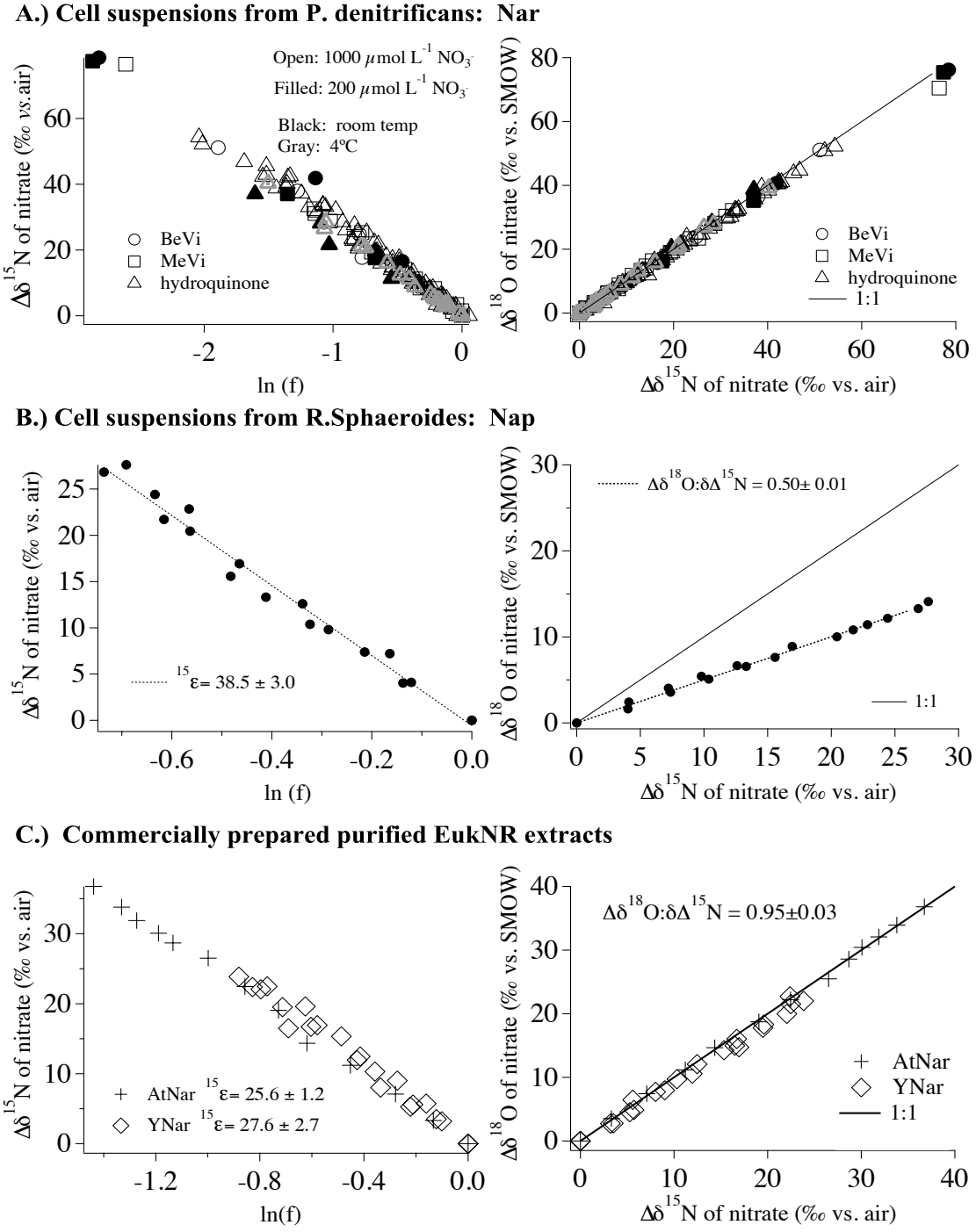

Figure 2: NO_3^- $\delta^{15}N$ vs ln [f] (where f is the fraction NO_3^- remaining: $[NO_3^-]$/ $[NO_3^-]$initial) [left panels] and the change in δ18O of NO_3^-vs the change in $\delta^{15}N$ of NO_3^- with 1:1 line shown for reference [right panels] for assays conducted with (a) cell suspensions of P. denitrificans, (b) cell suspensions of R. sphaeroides and (c) commercially prepared extracts of EukNR. The $\delta^{18}O$ and $\delta^{15}N$ are normalized to initial $\delta^{18}O$ and $\delta^{15}N$ values in all panels. The values for individual assays are reported in Table 1. In (a), shape represents reductant used (circle, square, and triangle for benzyl viologen, methyl viologen and hydroquinone, respectively). Shape fill represents $[NO_3^-]$ (open: 1000 μmol L^{-1}, shaded: 200 μmol L^{-1}) and color represents temperature at which the assay was conducted (black: room temperature, grey: 4°C). For (c), shape indicates type of commercially prepared EukNR; Crosses represent EukNR *f*rom *Arabidopsis thalania* (AtNar) and diamonds represent EukNR from the yeast *Pichia Angusta* (YNaR).

corresponding assays at higher temperature. Two hydroquinone-fuelled assays conducted with 200 μM [NO_3^-] produced isotope effects of 22.9 ± 1.5 and 25.6 ± 3.3 ‰.

For assays conducted with cell suspensions of *R. sphaeroides*, the magnitude of the change in $\delta^{15}N$ and $\delta^{18}O$ diverged during the [NO_3^-] drawdown, with $\delta^{15}N$ increasing to a greater extent than the corresponding $\delta^{18}O$ at a given [NO_3^-]. The magnitude of the N isotope effect averaged 37.4 ± 3.9‰ in two assays and the magnitude of the corresponding O isotope effect averaged 18.7 ± 1.9‰, resulting in a $\Delta\delta^{18}O:\Delta\delta^{15}N$ of 0.50 ± 0.01 (Table 2, Figure 2b).

Assays conducted with commercial stocks of purified EukNR from *Pichia angusta* (yeast-YNar) and *Arabidopsis thaliana* (AtNar) yielded N isotope effects ranging from 25.6 ± 1.1‰ to 28.0 ± 2.5‰. The $\Delta\delta^{18}O:\Delta\delta^{15}N$ was near 1, averaging of 0.96 ± 0.03 (Table 3, Figure 2c).

Discussion

N and O isotope behavior for nitrate reduction by the respiratory nitrate reductase, *Nar*

The O vs. N isotope coupling ($\Delta\delta^{18}O:\Delta\delta^{15}N$) among all anaerobic *P. denitrificans* cell suspension nitrate reductase assays was consistently on the order of 1 regardless of reductant type, initial nitrate concentration, or assay temperature. This confirms that *Nar*, the enzyme responsible for respiratory nitrate reduction by denitrifiers, fractionates the heavy N and O isotopologues of nitrate equivalently. The characteristic isotopic signal mirrors that observed in pure cultures of denitrifiers (Granger et al. 2008, Kritee et al. 2012, Wunderlich et al. 2012), corroborating unequivocally that bond breakage by the *Nar* nitrate reductase enzyme is the dominant fractionating step during respiratory denitrification (Granger et al. 2008). The coupling near unity is also consistent with that associated with water-column denitrification in marine systems (Sigman et al. 2003; 2005).

The average magnitudes of $^{15}\varepsilon$ (and $^{18}\varepsilon$) among all assays conducted with lysed cell suspensions of *P. denitrificans* were on the order of~27‰. The lower $^{15}\varepsilon$ and $^{18}\varepsilon$ values of ~6-10 ‰ observed with unlysed cell suspensions likely reflect incomplete equilibration of intracellular *vs.* external nitrate pools, thus dampening the propagation of the enzymatic isotope effect to the external buffer. The isotope effect observed for lysed cell suspensions, in contrast, ostensibly reflects the enzyme-level isotope effect, $^{15}\varepsilon_{Nar}$ and $^{18}\varepsilon_{Nar}$, unfettered by the effects of uptake and export of nitrate from a cell, which can serve to lower the observed isotope effect observed *in vivo*. The value of 27‰ observed here is in the general range of maximum isotope effects observed for denitrification in cultures (Wellman et al. 1968, Barford et al. 1999, Granger et al. 2008) and in in the environment (Brandes et al. 1998, Voss et al. 2001). A recent study, however, reported a more elevated $^{15}\varepsilon$ for *Nar* purified from *Escherichia. coli* of 31.6‰ using benzyl viologen as a reductant (Carlisle et al. 2014). This estimate, however, derived from a 2 point regression on the $\delta^{15}N$ of the nitrite product and is thus subject to considerable uncertainty. Nevertheless, there *are* reports of higher denitrification isotope effects *in vivo* (Wellman et al. 1968, Barford et al. 1999), including some observation of a $^{15}\varepsilon_{org}$ upwards of 31‰ for *P. denitrificans* grown in our laboratory (R. Dabundo, *personal communication*).

Some of our individual *Nar* assays with hydroquinone as a reductant yielded $^{15}\varepsilon$ and $^{18}\varepsilon$ equally elevated, although not consistently so. It is possible that the type of enzymatic reductant used in the assays could influence the magnitude of the enzyme level isotope effect, such as has been observed for nitrite reductase (Bryan et al. 1983). For a unidirectional enzyme-mediated reaction, the magnitude of the observed isotope effect depends on the degree to which the isotopically sensitive step of catalysis is rate-limiting. Enzyme mediated chemical reactions often involve multiple steps in addition to the chemical reaction itself. For example, *Nar*-

mediated nitrate reduction requires the succeeding reduction of three enzyme subunits as electrons are transferred from the quinol pool to the molybdenum active site of enzyme before the final reduction can take place. The speed of this electron transfer could affect the overall reaction rate of *Nar* and consequently that of the isotope effect.

Consider the irreversible enzymatic reaction outlined below:

$$E+S \underset{k_{back}}{\overset{k_{forward}}{\rightleftarrows}} ES \xrightarrow{k_{catalysis}} EP \qquad [1]$$

Once a substrate bonds with its enzyme at a specific reaction rate ($k_{forward}$) to form an enzyme-substrate complex it has one of two fates- either it is converted to product by the enzyme, also called catalysis at a specific reaction rate ($k_{catalysis}$; k_{cat}), or it is released from the enzyme at a specific reaction rate k_{back} and rejoins the substrate pool. Theoretically, the observed isotope effect is dependent on the relative reaction rates of catalysis and release [Eq. 2; O'Leary 1980, Karsh et al. 2012].

$$\varepsilon_{observed} = \frac{\varepsilon_{intrinsic} + k_{cat}/k_{back}}{1 + k_{cat}/k_{back}} \qquad [2]$$

where $\varepsilon_{intrisic}$ is the isotope effect associated with the catalytic step, quantified as ${}^{light}k_{cat}/{}^{heavy}k_{cat}$. If all substrate that binds is converted to product, the isotope effect will be zero, assuming no fractionation associated with binding; this occurs if the rate of catalysis (k_{cat}) is fast relative to the rate of unbinding (k_{back}), such that the 'commitment to catalysis,' k_{cat} / k_{back} , is large , dampening the expression of the intrinsic enzymatic isotope effect, $\varepsilon_{intrinsic}$ (Equation 1). At the other limit, when k_{back} is extremely fast relative to k_{cat}, the full intrinsic isotope effect $\varepsilon_{intrinsic}$ will be expressed in the residual substrate. In our experiments, k_{cat} for *Nar* was likely

modulated by the reductant type. The viologen reductants used here donate electrons directly to the molybdenum active site (Campbell 2001), whereas hydroquinone, which is the *in vivo* electron donor, donates to the cytochrome *b* subunit of *Nar*, requiring the electrons to sequentially reduce the Fe-sulfur clusters of the other two *Nar* subunits in turn before reaching the active site (Figure 3).

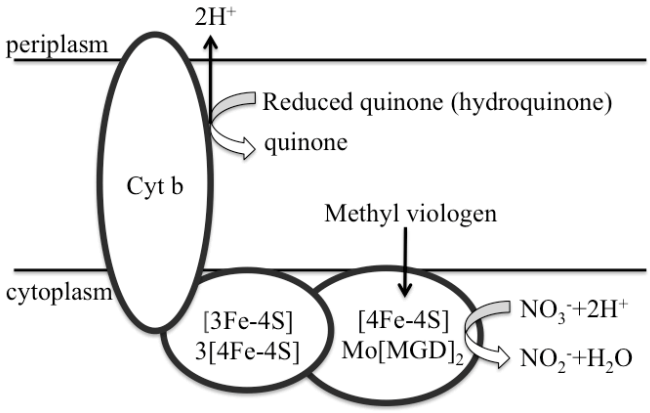

Figure 3: Schematic depicting the structure of dissimilatory nitrate reductase (*Nar*) and the locations of electron transfer by reductants hydroquinone (*in vivo* reductant) and benzyl or methyl viologen (artificial reductant). Cyt *b* denotes the transmembrane *b*-type cytochrome subunit, and 3Fe-4S and 4Fe-4S indicate iron-sulfur clusters of varying forms within both the secondary and catalytic subunit. Mo[MGD]2 denotes the molybdenum active site in the catalytic subunit. Figure reproduced from Berks et al. 1995.

Thus the use of artificial viologen reductants in lieu of hydroquinone, by speeding up k_{cat} relative to k_{back} and thereby decreasing the commitment to catalysis, could lower the isotope effect observed in the residual nitrate pool with methyl viologen compared to hydroquinone. This would suggest that the rate of internal electron transfer influences the overall enzymatic reaction rate, such that the isotopically sensitive step of N-O bond breakage is not entirely rate limiting in the enzymatic reaction. However, a higher N isotope effect was not observed consistently across all assays conducted with hydroquinone. It is possible the high isotope effect assays were a result of analytical error; however, this seems unlikely given that the nitrate

analyses and the isotopic analyses were conducted across multiple days with internal standards behaving as expected. Thus, it is unclear whether the observed isotope effect with either reductant is that intrinsic to bond breakage at the catalytic site of *Nar*, or whether the expression of the intrinsic isotope effect of *Nar* can be modulated as a function of the rate of internal electron transfer of the enzyme.

Perhaps even more confounding are the low N and O isotope effects observed for assays conducted with hydroquinone and 200 μmol L^{-1} [NO_3^-], of 22.9 ± 1.5 ‰ and 25.8 ± 3.3 ‰. While the latter $^{15}\varepsilon$ estimate is not significantly lower than that for corresponding assays conducted at 1000 μmol L^{-1} [NO_3^-] with hydroquinone, the former value is anomalously low. This could indicate an effect of [NO_3^-] on the enzymatic isotope effect, wherein lower substrate concentrations render substrate binding partially rate-determining relative to the catalytic rate, thereby reducing the proportion of substrate unbinding from the enzyme. However, we did not observe a reduced isotope effect for 200 μmol L^{-1} [NO_3^-] assays conducted with methyl or benzyl viologen, where this dynamic should be even more given the increase in k_{cat} promulgated by viologen. It is difficult to invoke a mechanism wherein [NO_3^-] affects the expression of the isotope effect for one reductant but not the other. Nevertheless, hydroquinone does seem to generally produce more variable isotope effect magnitudes.

Based on the above reasoning connecting the commitment to catalysis to the observed isotope effect, we might expect temperature to have an effect on the magnitude of the isotope effect, due to the temperature sensitivity of the rate of enzymatic reactions. We hypothesized that reducing temperature would slow k_{cat}, leading to an elevated k_{back}/ k_{cat} and thus an elevated isotope effect. However, assays conducted at 4ºC showed no significant difference in isotope effect from those at room temperature. It is possible the decrease in temperature did not impact

the catalytic rate sufficiently to create an observable difference in the isotope effect or that in addition to slowing k_{cat}, the reduction in temperature slowed k_{back} proportionally such that the relative rates of the two processes did not change significantly with the temperature decrease and the commitment to catalysis remained roughly the same. This result is surprising, however, given the possible sensitivity of commitment to catalysis and thus the isotope effect to changes in k_{cat} brought about by reductant.

Although the work of Karsh et al. (2012) strongly suggests an invariant isotope effect near 27‰ for nitrate reduction by EukNR, the authors recognized the possibility of its sensitivity to reductant type by way of its effect on k_{cat}. Their assays were conducted using methyl viologen, and they were not able to directly test the isotope effect using the *in vivo* electron donors NADH and NAD(P)H due to their incompatibility with the nitrite removal method with sulfamic acid (Granger and Sigman 2009). Although they expected a slower k_{cat} with NADH and NAD[P]H, based on the magnitude of the N isotope effect observed for assimilation in cultures of the same algal species (*T. weissflogi* ε_{org}<23.5 ‰: Needoba et al. 2004) and in one nitrate reduction assay study with EukNR from spinach leaves and NAD[P]H ($\varepsilon_{NAD[P]H}$=15‰: Ledgard et al. 1985) they inferred that for EukNR, $\varepsilon_{NAD[P]H} \sim \varepsilon_{MeVi}$. In order for the rate of electron transfer to not influence the magnitude of the isotope effect, they speculated that either NO_3^- would only bind to a reduced molybdenum center that had already received electrons and thus electron transfer rate is irrelevant or that NO_3^- binds to an oxidized Mo center but the substrate is in a state of rapid equilibrium with the enzyme and thus the rate of dissociation (k_{back})>> rate of catalysis (k_{cat}). This leads to a commitment to catalysis approaching zero and thus an observed isotope effect equal to that of the intrinsic isotope effect for N-O bond rupture regardless of electron transfer rate. Our results for *Nar*, however, indicate that $\varepsilon_{hydroquinone}$ may be greater than ε_{MeVi} under some

conditions, which suggests both that NO_3^- is binding with an oxidized Mo center and that substrates are not in rapid equilibrium with the enzyme, causing rate of electron transfer to affect commitment to catalysis and thus the observed isotope effect. Results where $\varepsilon_{hydroquinone} < \varepsilon_{MeVi}$ are, however, difficult to reconcile under this scenario and suggest a degree of malleability to ε_{Nar} that cannot be completely explained by internal rate of electron transfer and commitment to catalysis.

N and O isotope behavior for nitrate reduction by periplasmic nitrate reductase, *Nap*

In contrast to *Nar*, the periplasmic dissimilatory nitrate reductase, *Nap*, assayed from *Rhodobacter sphaeroides* cell suspensions does not fractionate N and O in a ratio near unity, but rather, with a $\Delta\delta^{18}O$: $\Delta\delta^{15}N$ on the order of 0.5. This trend resembles that observed in a previous study during nitrate drawdown in cultures of the same strain of *R. sphaeroides*, with a $\Delta\delta^{18}O$: $\Delta\delta^{15}N \cong 0.6$ (Granger et al. 2008), albeit slightly lower. This difference is difficult to reconcile, given the high precision and accuracy of the N and O isotope ratio measurements with the denitrifier method. The discrepancy could reflect different strains in spite of appearances to the contrary, differences imparted by incomplete nitrite removal (although unlikely), or from nitrite reoxidation during prolonged storage of samples (in the case of Granger et al. 2008). Nevertheless, the $\Delta\delta^{18}O$: $\Delta\delta^{15}N$ of 0.5 observed here is analogous to that observed recently in cultures of the autotrophic Epsilon-protoebacterium, *S. gotlandica*, which oxidizes sulfide for autotrophic carbon fixation while using nitrate as an electron donor *via* the periplasmic *Nap* nitrate reductase (Frey et al. 2014). The unique $\Delta\delta^{18}O$: $\Delta\delta^{15}N$ coupling of *Nap* affirmed in two distantly related strains clearly suggests there is something fundamentally different about the way in which *Nap* binds with nitrate or reduces nitrate compared to the other nitrate reducing

enzymes, despite the structural and functional similarities of *Nap* to *Nar* enzymes. It is puzzling that *Nap* fractionates N and O differently even though it performs the same chemical function as both *Nar* and EukNR, the N-O bond rupture during nitrate reduction. The isotopic signal should theoretically reflect the energetic difference of the breaking bonds for a 'heavy' vs. a 'light' nitrate molecule bound to a hexadentate molybdenum atom, and therefore should be the similar for all nitrate reductases, given a similar binding configuration of nitrate to the Mo atom. Furthermore, *Nap* is more closely related to *Nar* genetically than *Nar* is to EukNR (Richardson et al. 2001), although both of the latter fractionate N and O analogously. *Nap* and *Nar* are both classified as part of the DMSO family of molybdoenzymes, but differ marginally in their coordination of the Mo active sites; both are coordinated by two molybdopterin guanidine dinucleotide (MGD) molecules providing two bidentate dithiolene ligands. The coordination of *Nap*'s Mo atom is completed with a cysteine or selenium-cysteine ligand, whereas *Nar*'s is completed with an asparginine ligand (Moreno-Vivian et al. 1999, Sparacino-Watkins et al. 2014). Both also possess additional oxo/hydroxo/ water ligands While their coordination sites are similar, *Nap* has two enzyme subunits to *Nar*'s three; it possesses a catalytic subunit with a MGD-cofactor and [4Fe-4S] cluster and a *c-type* cytochrome whereas *Nar* has a soluble subunit consisting of three [4Fe-4S] clusters and one [3Fe-4S] cluster and a membrane bound bi-heme cytochrome *b* subunit that receives electrons from the quinol pool in addition to the catalytic subunit with an MGD-cofactor and [4Fe-4S] cluster (Moreno-Vivian et al 1999). The active site of *Nap* is reportedly specific to nitrate, but *Nar*'s is relatively nonspecific and has been observed binding with other mono-charged anions such as fluoride, nitrite, formate, chlorate and bromate (George et al., 1985, Jormakka et al. 2004). How these differences could translate to differences

in the ratio of O to N isotope effects, however, remains unclear and is beyond the scope of this project.

The magnitude of the enzymatic N isotope effect of *Nap* observed here (38.5 ± 3.0 ‰) is significantly higher than that observed for both Nar and EukNR. Like the deviation from $\Delta\delta^{18}O$: $\Delta\delta^{15}N \cong 1$, the high magnitude of the N isotope effect further suggests there is something fundamentally different about the mechanism by which *Nap* reduces nitrate. Because the reaction rate difference between the heavy and light molecule is greater when *Nap* facilitates reduction, the energy difference between a ^{15}N-^{16}O-Mo and a ^{14}N-^{16}O-Mo bond must be greater when *Nap* binds the enzyme compared with the other nitrate reductases. Given the context of the greater specificity for substrate of *Nap* compared to *Nar*, this perhaps suggests a more complex binding interaction of the nitrate molecule with the enzyme's reaction center. The oxygen isotope effect, however, is lower for *Nap* than for *Nar*, which perhaps indicates *Nap* does not bind to an ^{18}O and only interacts with the ^{16}Os on a $^{14}N^{16}O^{16}O^{18}O$ nitrate molecule or if it does, the ^{18}O-Mo bond has a favorable energy which makes N-O bond breakage slightly more favorable than breaking the ^{18}O-Mo bond of the enzyme bound transition state.

Nap is unique in that the physiological purpose served by its nitrate reduction differs between organisms and in different growth conditions (Hartsock and Shapleigh 2011). Because it does not generate a proton gradient across the cell membrane, nitrate reduction *via Nap* is generally cited as not directly coupled to energy production as it is for *Nar* (Sparacino-Watkins et al. 2014). In some cases, *Nap* is thought to act as means of disposing of excess reducing power through the reduction of nitrate (Richardson et al. 2001, Ellington et al. 2002, Gavira et al. 2002). This is especially important for redox balancing during growth on reduced carbon sources and during photoheterotrophic growth, for which the turnover of the photosystem depends on the

supply of oxidized quinone from the quinone/quinol pool (reviewed by Berks et al. 1995). The product nitrite can then too, be used downstream in the remainder of the denitrification pathway for respiratory purposes (Bedzyk et al. 1999, Bedmar et al. 2005) including for dissimilatory nitrate reduction to ammonium (DNRA). In certain strains, including *P. denitrificans*, *Nap* has also been shown to be preferentially expressed under aerobic growth conditions (Gavira et al. 2002). And as in other organisms who possess both *Nap* and *Nar*, *Nap* in *P. denitrificans* has been shown to be required for transition to anaerobeosis, facilitating denitrification under conditions where the expression of *Nar* or transport of nitrate into the cytoplasm would be inhibited by the presence of oxygen (Bedzyk et al. 1999, Alefounder and Ferguson 1980, Moir and Wood 2001). Because of this, we hypothesized that assays conducted with cell suspensions of aerobically grown *P. denitrificans* would result in a $\Delta\delta^{18}O$: $\Delta\delta^{15}N < 1$, reflecting the activity of *Nap*; however, these assays showed no difference from assays with anaerobically grown *P. denitrificans* cell suspensions. Both fractionated N and O equivalently. This suggests either the *Nar* in our assays was active even though the cells were grown aerobically, or that catalysis by the *P. denitrificans'* Nap fractionates with a $\Delta\delta^{18}O$: $\Delta\delta^{15}N$ of 1, and thus differs from catalysis by *Nap* in *R. sphaeroides* or in *S. gotlandica*. Although this latter scenario seems unlikely, this is not impossible given a comparatively high degree of functional and genetic diversity within the *Nap* group of nitrate reductases, compared to *Nar* or EukNR which are more highly conserved (Richardson et al. 2001, Hartsock and Shapleigh 2011).

The magnitude of the N isotope effect of 37.4‰ associated with *R. sphaeroides Nap* is much greater than the magnitude observed *in vivo* in cultures of *R. sphaeroides* and of *S. gotlandica*, where N isotope effects were on the order ~15‰ and 23.8 ± 2.5‰ respectively. While for *Nar* and EukNR, the discrepancy between the enzymatic isotope effect and that observed in culture or

the environment is expected due to the diluting effects of uptake and export on the enzymatic isotope effect imparted internally, we anticipated less of a difference in isotope amplitude for *Nap*, whose location in the periplasm of the cell we hypothesized should isolate it from the effects associated with active membrane transport and enable homogenization of the periplasmic pool with the external nitrate pool. Therefore, this result is surprising, and even more so because the discrepancy is greater than that between the magnitude of the N isotope effect of cultures of denitrifying bacteria and *Nar*, whose location at the cell interior renders the expression of its isotope effect subject to the effects of membrane transport. In previous studies, diffusion limitation across a cell boundary layer has been discussed as a mechanism that could serve to lower the isotope effect of denitrifiers but has been disregarded based on the reasoning that bacterial cells are too small and nitrate concentrations too high in these studies (>1 mmol L^{-1}) to result in a diffusive boundary layer (Kritee et al. 2012, Pasciack and Gavis 1974, Frey at al. 2014). Interestingly, Frey et al (2014) reported a higher isotope effect for cultures of *S. gotlandica* that were shaken during growth *vs.* stationary cultures. They attributed this result to diffusion limitation imposed by a vertical [NO_3^-] gradient within the media arguing that dense cells settled near the bottom consumed nitrate more rapidly than those at the top. This would result in a gradient of $\delta^{15}N$ and $\delta^{18}O$ with greater values near the bottom coincident with a higher degree of nitrate consumption. However, as our $^{15}\varepsilon$ for Nap (38.5 ‰) was higher than their high $^{15}\varepsilon$ for shaken cultures (19.4-27.7 ‰) and considerably higher than that observed *in vivo* for non-shaken cultures (13.2 –18.4 ‰), a different or additional mechanism is required to explain the low isotope effect observed in cultures. We suggest the possibility of a mixing limitation of enriched residual substrate in the periplasmic space with external media. Although porin channels in the outer membrane of gram negative bacteria allow the free diffusion of small

hydrophilic molecules such as NO_3^- in and out of the periplasmic space (Galdiero et al. 2012), it is likely that there is incomplete homogenization between the periplasmic and external nitrate pools of roughly equal chemical but not isotopic composition, leading to an under expression of the enzymatic isotope effect in the external media. Shaking may thus affect a greater exchange of the periplasmic and external nitrate pool.

N and O isotope behavior for nitrate reduction by eukaryotic assimilatory nitrate reductase EukNR

The commercially prepared pure extracts of recombinant EukNR from *Arabidopsis thalania* and *Pichia angusta* both yielded a $\Delta\delta^{18}O$: $\Delta\delta^{15}N \cong 1$. These results are consistent with analogous measurement of Karsh et al. 2012, who also observed a coupling near unity for EukNR isolated for *Aspergillus* sp., and in cell homogenates of the diatom *T. weissflogii*. The magnitude of the N (and O) isotope effect averaged 27‰, also identical to the magnitude observed by Karsh et al. (2012). Our results, however, contradict a recent report of a $^{15}\varepsilon$ for recombinant EukNR from *P. angusta* procured from the same supplier, which were on the order of 22‰ (Carlisle et al. 2014). We expected to replicate this result, hypothesizing the discrepancy of $^{15}\varepsilon$ amplitude between that and other nitrate reductases could be due to the preparation of the enzyme extracts, which were expressed recombinantly in *Pichia pastoris* and missing parts of the native enzyme. However, our results refute this, and rather suggest an isotope effect of 27‰ for this enzyme as for other eukaryotic nitrate reductases, at least when fuelled by methyl viologen. The $^{15}\varepsilon$ measured by Carlisle et al. (2014) likely owes to a methodological difference; the isotopic effect calculated therein is based on a Rayleigh linear fit of only two N isotopic measures (beginning and endpoint) of the nitrite product. Because this method relies on only two

data points, an under or overestimate of either could easily lead to a miscalculation of the isotope effect.

Both the $\Delta\delta^{18}O$: $\Delta\delta^{15}N$ coupling and amplitude of the N and O isotope effects observed among EukNR enzymes is identical to that measured here for *Nar*. This clearly owes to functional and structural similarity between these enzymes; both reduce nitrate to nitrite and both are mononuclear, hexadentate molybdoenzymes. Nevertheless, EukNR is in the sulfite oxidase family of molybdoenzymes and is bound to a single molybdopterin (MPT) moiety (Campbell, 1999), whereas *Nar* belongs to the dimethyl sulfoxide oxidase (DMSO) reductase family due to coordination of the Mo active site by a bis-molybdopterin guanine dinucleotide (MGD; Berks et al. 1995). The two reductase types, *Nar* and EukNR, further belong to distinct genetic clades wherein *Nar* is a distant relative of the monophyletic EukNR enzymes (Stolz and Basu 2002). In this respect, the isotopic similarities are remarkable.

Conclusions

Our results demonstrate that *Nar*, like EukNR, imparts a consistent coupling on the O and N isotopologues of nitrate, where $\Delta\delta^{18}O$: $\Delta\delta^{15}N$ is on the order of 1. The $^{15}\varepsilon$ (and $^{18}\varepsilon$) of both *Nar* and EukNR prove to be identical, at least when fuelled by viologen. Theoretically, and based on the variability incurred when quinone as reductant, we posit that a higher enzymatic isotope effect is plausible *in vivo*, whereas a lower isotope effect is difficult to reconcile based on our understanding of the enzymatic mechanism. Nevertheless, the elevated $^{15}\varepsilon$ may represent a hypothetical upper limit of attainable isotope effects in vivo for both denitrification and nitrate assimilation.

The distinctive $\Delta\delta^{18}O$: $\Delta\delta^{15}N$ signature of 1 associated with respiratory denitrification and nitrate assimilation provides a benchmark for environmental studies, wherein biological nitrate consumption can be identified from nitrate isotope distributions, and distinguished from co-occurring N transformations. Our data further discredit the notion that the O-N coupling of denitrification is variable, which has be suggested by some (Knöller et al. 2011). Thus, by itself, the coupling of unity fails to explain the coupling of 0.5 to 0.7 observed in association with N loss in aquifers and lakes. We suggest that the coupling below 1 is indicative of nitrate production, by nitrification or anaerobic ammonium oxidation (anammox), co-incident with denitrification, thus overprinting the $\Delta\delta^{18}O$: $\Delta\delta^{15}N$ of 1.

It has also been argued that nitrate reduction mediated by *Nap*, which results in a nitrate reduction signal with a $\Delta\delta^{18}O$: $\Delta\delta^{15}N < 1$, could drive the signal observed in freshwater systems. While a facile explication, it requires that *Nap* effectuate the majority of nitrate reduction in aquifers and lakes, which seems difficult reconcile with the role of *Nap* as an auxiliary reductase involved in redox balancing. Moreover, it proved difficult to silence the $\Delta\delta^{18}O$: $\Delta\delta^{15}N$ of 1 for *P. denitrificans* cultures in this study, even when grown in conditions that favor the expression of *Nap*. We cannot rule out the possibility of environmental conditions where *Nap* dominates the nitrate reduction signal, such as in niches where the microbial community is dominated by autotrophic sulfate oxidizing bacteria that possess only *Nap* (see Wenk et al. 2014; Frey et al. 2014). However, the denitrification signal in most environments likely mediated by *Nar* and thus propagates a $\Delta\delta^{18}O$: $\Delta\delta^{15}N$ of 1 to ambient nitrate.

References

Alefounder, P.R., and Ferguson, S.J., 1980, The location of dissimilatory nitrite reductase and the control of dissimilatory nitrate reductase by oxygen in *Paracoccus denitrificans*. Biochem.J, v. 192, p. 231-240.

Altabet, M.A., and Francois, R., 2001, Nitrogen isotope biogeochemistry of the Antarctic Polar Frontal Zone at 170 W: Deep Sea Research Part II: Topical Studies in Oceanography, v. 48, p. 4247-4273.

Altabet, M.A., and Curry, W.B., 1989, Testing models of past ocean chemistry using foraminifera 15N/14N: Global Biogeochemical Cycles, v. 3, p. 107-119, doi: 10.1029/GB003i002p00107.

Amberger, A., and Schmidt, H., 1987, Natürliche isotopengehalte von Nitrat als Indikatoren für dessen Herkunft: Geochimica Et Cosmochimica Acta, v. 51, p. 2699-2705.

Barford, C.C., Montoya, J.P., Altabet, M.A., and Mitchell, R., 1999, Steady-state nitrogen isotope effects of N_2 and N_2O production in *Paracoccus denitrificans*: Applied and Environmental Microbiology, v. 65, p. 989-994.

Bedmar, E., Robles, E., and Delgado, M., 2005, The complete denitrification pathway of the symbiotic, nitrogen-fixing bacterium *Bradyrhizobium japonicum*: Biochemical Society Transactions, v. 33, p. 141-144.

Bedzyk, L., Wang, T., and Ye, R.W., 1999, The periplasmic nitrate reductase in *Pseudomonas sp.* strain G-179 catalyzes the first step of denitrification: Journal of Bacteriology, v. 181, p. 2802-2806.

Bender, R.A., and Friedrich, B., 1990, Regulation of assimilatory nitrate reductase formation in *Klebsiella aerogenes* W70: Journal of Bacteriology, v. 172, p. 7256-7259.

Berks, B.C., Ferguson, S.J., Moir, J.W., and Richardson, D.J., 1995, Enzymes and associated electron transport systems that catalyse the respiratory reduction of nitrogen oxides and oxyanions: Biochimica et Biophysica Acta (BBA)-Bioenergetics, v. 1232, p. 97-173.

Böhlke, J., Mroczkowski, S., and Coplen, T., 2003, Oxygen isotopes in nitrate: New reference materials for ^{18}O: ^{17}O: ^{16}O measurements and observations on nitrate-water equilibration: Rapid Communications in Mass Spectrometry, v. 17, p. 1835-1846.

Böttcher, J., Strebel, O., Voerkelius, S., and Schmidt, H., 1990, Using isotope fractionation of nitrate-nitrogen and nitrate-oxygen for evaluation of microbial denitrification in a sandy aquifer: Journal of Hydrology, v. 114, p. 413-424.

Braman, R.S., and Hendrix, S.A., 1989, Nanogram nitrite and nitrate determination in environmental and biological materials by vanadium (III) reduction with chemiluminescence detection: Analytical Chemistry, v. 61, p. 2715-2718.

Brandes, J.A., and Devol, A.H., 2002, A global marine-fixed nitrogen isotopic budget: Implications for Holocene nitrogen cycling: Global Biogeochemical Cycles, v. 16, p. 67-1-67-14.

Brandes, J.A., Devol, A.H., Yoshinari, T., Jayakumar, D., and Naqvi, S., 1998, Isotopic composition of nitrate in the central Arabian Sea and eastern tropical North Pacific: A tracer for mixing and nitrogen cycles: Limnology and Oceanography, v. 43, p. 1680-1689.

Bryan, B.A., Shearer, G., Skeeters, J.L., and Kohl, D.H., 1983, Variable expression of the nitrogen isotope effect associated with denitrification of nitrite: The Journal of Biological Chemistry, v. 258, p. 8613-8617.

Buchwald, C., and Casciotti, K.L., 2010, Oxygen isotopic fractionation and exchange during bacterial nitrite oxidation: Limnology and Oceanography, v. 55, p. 1064-1074.

Campbell, W., 2001, Structure and function of eukaryotic NAD (P) H: nitrate reductase: Cellular and Molecular Life Sciences CMLS, v. 58, p. 194-204.

Campbell, W.H., 1999, Nitrate reductase structure, function and regulation: bridging the gap between biochemistry and physiology: Annual Review of Plant Biology, v. 50, p. 277-303.

Canfield, D.E., Glazer, A.N., and Falkowski, P.G., 2010, The evolution and future of Earth's nitrogen cycle: Science,, v. 330, p. 192-196, doi: 10.1126/science.1186120 [doi].

Carlisle, E., Yarnes, C., Toney, M.D., and Bloom, A.J., 2014, Nitrate reductase 15N discrimination in *Arabidopsis thaliana*, *Zea mays*, *Aspergillus niger*, *Pichea angusta*, and *Escherichia coli*: Frontiers in Plant Science, v. 5, .

Casciotti, K.L., Buchwald, C., and McIlvin, M., 2013, Implications of nitrate and nitrite isotopic measurements for the mechanisms of nitrogen cycling in the Peru oxygen deficient zone: Deep Sea Research Part I: Oceanographic Research Papers, v. 80, p. 78-93.

Casciotti, K., and McIlvin, M., 2007, Isotopic analyses of nitrate and nitrite from reference mixtures and application to Eastern Tropical North Pacific waters: Marine Chemistry, v. 107, p. 184.

Casciotti, K.L., Sigman, D.M., Hastings, M.G., Bohlke, J.K., and Hilkert, A., 2002, Measurement of the Oxygen Isotopic Composition of Nitrate in Seawater and Freshwater Using the Denitrifier Method: Analytical Chemistry, v. 74, p. 4905-4912, doi: 10.1021/ac020113w.

Cline, J., and Kaplan, I., 1975, Isotopic fractionation of dissolved nitrate during denitrification in the eastern tropical North Pacific Ocean: Marine Chemistry, v. 3, p. 271-299.

Codispoti, L.A., 1995, Is the ocean losing nitrate?: Nature, v. 376, p. 724.

Deutsch, C., Sarmiento, J., Sigman, D., Gruber, N., and Dunne, J., 2007, Spatial coupling of nitrogen inputs and losses in the ocean: Nature, v. 445, p. 163.

DiFiore, P.J., Sigman, D.M., and Dunbar, R.B., 2009, Upper ocean nitrogen fluxes in the Polar Antarctic Zone: Constraints from the nitrogen and oxygen isotopes of nitrate: Geochemistry, Geophysics, Geosystems, v. 10, .

DiFiore, P.J., Sigman, D.M., Trull, T.W., Lourey, M.J., Karsh, K., Cane, G., and Ho, R., 2006, Nitrogen isotope constraints on subantarctic biogeochemistry: Journal of Geophysical Research: Oceans (1978–2012), v. 111, .

Ellington, M.J., Bhakoo, K.K., Sawers, G., Richardson, D.J., and Ferguson, S.J., 2002, Hierarchy of carbon source selection in *Paracoccus pantotrophus*: strict correlation between reduction state of the carbon substrate and aerobic expression of the nap operon: Journal of Bacteriology, v. 184, p. 4767-4774.

Falkowski, P.G., 1997, Evolution of the nitrogen cycle and its influence on the biological sequestration of CO_2 in the ocean: Nature, v. 387, p. 272-275.

Francois, R., Altabet, M.A., Goericke, R., McCorkle, D.C., Brunet, C., and Poisson, A., 1993, Changes in the δ13C of surface water particulate organic matter across the subtropical convergence in the SW Indian Ocean: Global Biogeochemical Cycles, v. 7, p. 627-644.

Frey, C., Hietanen, S., Jürgens, K., Labrenz, M., and Voss, M., 2014, N and O isotope fractionation in nitrate during chemolithoautotrophic denitrification by *Sulfurimonas gotlandica*: Environmental Science & Technology, v. 48, p. 13229-13237.

Galdiero, S., Falanga, A., Cantisani, M., Tarallo, R., Della Pepa, M.E., D'Oriano, V., and Galdiero, M., 2012, Microbe-host interactions: structure and role of Gram-negative bacterial porins: Current Protein & Peptide Science, v. 13, p. 843-854, doi: CPPS-EPUB-20121210-11 [pii].

Garside, C., 1982, A Chemiluminescent technique for the determination of nanomolar concentrations of nitrate and nitrite in seawater: Marine Chemistry, v. 11, p. 159.

Gavira, M., Roldan, M.D., Castillo, F., and Moreno-Vivian, C., 2002, Regulation of nap gene expression and periplasmic nitrate reductase activity in the phototrophic bacterium *Rhodobacter sphaeroides* DSM158: Journal of Bacteriology, v. 184, p. 1693-1702.

George, G.N., Bray, R., Morpeth, F., and Boxer, D., 1985, Complexes with halide and other anions of the molybdenum centre of nitrate reductase from *Escherichia coli*. Biochem.J, v. 227, p. 925-931.

Gonfiantini, R., Stichler, W., and Rozanski, K., 1995, Standards and intercomparison materials distributed by the International Atomic Energy Agency for stable isotope measurements: .

Granger, J., Sigman, D.M., Prokopenko, M.G., Lehmann, M.F., and Tortell, P.D., 2006, A method for nitrite removal in nitrate N and O isotope analyses: Limnology and Oceanography: Methods, v. 4, p. 205-212.

Granger, J., Sigman, D., Lehmann, M., and Tortell, P., 2008, Nitrogen and oxygen isotope fractionation during dissimilatory nitrate reduction by denitrifying bacteria: Limnology and Oceanography, v. 53, p. 2533.

Granger, J., Sigman, D.M., Rohde, M.M., Maldonado, M.T., and Tortell, P.D., 2010, N and O isotope effects during nitrate assimilation by unicellular prokaryotic and eukaryotic plankton cultures: Geochimica et Cosmochimica Acta, v. 74, p. 1030-1040, doi: DOI: 10.1016/j.gca.2009.10.044.

Granger, J., and Sigman, D.M., 2009, Removal of nitrite with sulfamic acid for nitrate N and O isotope analysis with the denitrifier method: Rapid Communications in Mass Spectrometry, v. 23, p. 3753-3762, doi: 10.1002/rcm.4307.

Granger, J., Sigman, D.M., Needoba, J.A., and Harrison, P.J., 2004, Coupled Nitrogen and Oxygen Isotope Fractionation of Nitrate during Assimilation by Cultures of Marine Phytoplankton: Limnology and Oceanography, v. 49, p. 1763-1773.

Gruber, N., 2008, The marine nitrogen cycle: overview and challenges: Nitrogen in the Marine Environment, p. 1-50.

Gruber, N., and Galloway, J.N., 2008, An Earth-system perspective of the global nitrogen cycle: Nature, v. 451, p. 293-296.

Gruber, N., and Sarmiento, J.L., 1997, Global patterns of marine nitrogen fixation and denitrification: Global Biogeochemical Cycles, v. 11, p. 235-266.

Guillard, R.R., 1975, Culture of phytoplankton for feeding marine invertebrates, *in* Culture of marine invertebrate animals: Springer, p. 29-60.

Hartsock, A., and Shapleigh, J.P., 2011, Physiological roles for two periplasmic nitrate reductases in *Rhodobacter sphaeroides* 2.4.3 (ATCC 17025): Journal of Bacteriology, v. 193, p. 6483-6489, doi: 10.1128/JB.05324-11 [doi].

Jepson, B.J.N., Anderson, L.J., Rubio, L.M., Taylor, C.J., Butler, C.S., Flores, E., Herrero, A., Butt, J.N., and Richardson, D.J., 2004, Tuning a Nitrate Reductase for Function: The first spectropotentiometric characterization of a bacterial assimilatory nitrate reductase reveals novel redox properties: Journal of Biological Chemistry, v. 279, p. 32212-32218, doi: 10.1074/jbc.M402669200.

Jormakka, M., Richardson, D., Byrne, B., and Iwata, S., 2004, Architecture of NarGH reveals a structural classification of Mo-bisMGD enzymes: Structure, v. 12, p. 95-104.

Karsh, K., Trull, T., Sigman, D., Thompson, P., and Granger, J., 2014, The contributions of nitrate uptake and efflux to isotope fractionation during algal nitrate assimilation: Geochimica Et Cosmochimica Acta, v. 132, p. 391-412.

Karsh, K.L., Granger, J., Kritee, K., and Sigman, D.M., 2012, Eukaryotic assimilatory nitrate reductase fractionates N and O isotopes with a ratio near unity: Environmental Science & Technology, v. 46, p. 5727-5735.

Knöller, K., Vogt, C., Haupt, M., Feisthauer, S., and Richnow, H., 2011, Experimental investigation of nitrogen and oxygen isotope fractionation in nitrate and nitrite during denitrification: Biogeochemistry, v. 103, p. 371-384.

Korner, H., and Zumft, W.G., 1989, Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of *Pseudomonas stutzeri*: Applied and Environmental Microbiology, v. 55, p. 1670-1676.

Kritee, K., Sigman, D.M., Granger, J., Ward, B.B., Jayakumar, A., and Deutsch, C., 2012, Reduced isotope fractionation by denitrification under conditions relevant to the ocean: Geochimica Et Cosmochimica Acta, v. 92, p. 243-259.

Ledgard, S., Woo, K., and Bergersen, F., 1985, Isotopic fractionation during reduction of nitrate and nitrite by extracts of spinach leaves: Functional Plant Biology, v. 12, p. 631-640.

Lehmann, M.F., Reichert, P., Bernasconi, S.M., Barbieri, A., and McKenzie, J.A., 2003, Modelling nitrogen and oxygen isotope fractionation during denitrification in a lacustrine redox-transition zone: Geochimica Et Cosmochimica Acta, v. 67, p. 2529-2542.

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

Moir, J., and Wood, N., 2001, Nitrate and nitrite transport in bacteria: Cellular and Molecular Life Sciences CMLS, v. 58, p. 215-224.

Montoya, J.P., and McCarthy, J.J., 1995, Isotopic fractionation during nitrate uptake by phytoplankton grown in continuous culture: Journal of Plankton Research, v. 17, p. 439-464.

Moreno-Vivian, C., Cabello, P., Martinez-Luque, M., Blasco, R., and Castillo, F., 1999, Prokaryotic nitrate reduction: molecular properties and functional distinction among bacterial nitrate reductases: Journal of Bacteriology, v. 181, p. 6573-6584.

Needoba, J.A., Sigman, D.M., and Harrison, P.J., 2004, The mechanism of isotope fractionation during algal nitrate assimilation as illuminated by 15N/14N of intracellular nitrate: Journal of Phycology, v. 40, p. 517-522.

Needoba, J., Sigman, D., and Harrison, P., 2004, The mechanism of isotope fractionation during algal nitrate assimilation as illuminated by the N-15/N-14 of intracellular nitrate: Journal of Phycology, v. 40, p. 517.

Needoba, J., Waser, N., Harrison, P., and Calvert, S., 2003, Nitrogen isotope fractionation in 12 species of marine phytoplankton during growth on nitrate: Marine Ecology Progress Series, v. 255, p. 81.

O'Leary, M.H., 1980, Determination of heavy-atom isotope effects on enzyme-catalyzed reactions: Methods in Enzymology, v. 64, p. 83-104.

Pasciak, W.J., and Gavis, J., 1974, Transport limitation of nutrient uptake in phytoplankton1: Limnology and Oceanography, v. 19, p. 881-888.

Richardson, D., Berks, B., Russell, D., Spiro, S., and Taylor, C., 2001, Functional, biochemical and genetic diversity of prokaryotic nitrate reductases: Cellular and Molecular Life Sciences CMLS, v. 58, p. 165-178.

Schmidt, H., and Medina, R., 1991, Possibilities and scope of the double isotope effect method in the elucidation of mechanisms of enzyme catalyzed reactions: Isotopenpraxis Isotopes in Environmental and Health Studies , v. 27, p. 1-4.

Sigman, D.M., DiFiore, P.J., Hain, M.P., Deutsch, C., Wang, Y., Karl, D.M., Knapp, A.N., Lehmann, M.F., and Pantoja, S., 2009, The dual isotopes of deep nitrate as a constraint on the cycle and budget of oceanic fixed nitrogen: Deep Sea Research Part I: Oceanographic Research Papers, v. 56, p. 1419-1439.

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

Sigman, D., Altabet, M., McCorkle, D., Francois, R., and Fischer, G., 1999, The δ15N of nitrate in the Southern Ocean: consumption of nitrate in surface waters: Global Biogeochemical Cycles, v. 13, p. 1149-1166.

Sigman, D., Altabet, M., Michener, R., McCorkle, D., Fry, B., and Holmes, R., 1997, Natural abundance-level measurement of the nitrogen isotopic composition of oceanic nitrate: an adaptation of the ammonia diffusion method: Marine Chemistry, v. 57, p. 227-242.

Sigman, D., Karsh, K., and Casciotti, K., 2009, Ocean process tracers: nitrogen isotopes in the ocean: Encyclopedia of Ocean Science, 2nd Edn.Elsevier, Amsterdam, .

Sigman, D., Robinson, R., Knapp, A., Van Geen, A., McCorkle, D., Brandes, J., and Thunell, R., 2003, Distinguishing between water column and sedimentary denitrification in the Santa Barbara Basin using the stable isotopes of nitrate: Geochemistry, Geophysics, Geosystems, v. 4, .

Sigman, D., Casciotti, K., Andreani, M., Barford, C., Galanter, M., and Bohlke, J., 2001, A bacterial method for the nitrogen isotopic analysis of nitrate in seawater and freshwater: Analytical Chemistry, v. 73, p. 4145.

Sokal, R.R., and Rohlf, F.J., 1995, The principles and practice of statistics in biological research: WH Freeman and Co.: New York, .

Sparacino-Watkins, C., Stolz, J.F., and Basu, P., 2014, Nitrate and periplasmic nitrate reductases: Chemical Society Reviews, v. 43, p. 676-706.

Stolz, J.F., and Basu, P., 2002, Evolution of nitrate reductase: molecular and structural variations on a common function: Chembiochem, v. 3, p. 198-206.

Voss, M., Dippner, J.W., and Montoya, J.P., 2001, Nitrogen isotope patterns in the oxygen-deficient waters of the Eastern Tropical North Pacific Ocean: Deep Sea Research Part I: Oceanographic Research Papers, v. 48, p. 1905-1921.

Wada, E., 1980, Nitrogen isotope fractionation and its significance in biogeochemical processes occurring in marine environments: Isotope Marine Chemistry, v. 1, p. 375-398.

Wada, E., and Hattori, A., 1978, Nitrogen isotope effects in the assimilation of inorganic nitrogenous compounds by marine diatoms: Geomicrobiology Journal, v. 1, p. 85-101.

Wada, E., Kadonaga, T., and Matsuo, S., 1975, 15N abundance in nitrogen of naturally occurring substances and global assessment of denitrification from isotopic viewpoint: Geochemical Journal, v. 9, p. 139-148.

Waser, N., Harrison, P., Nielsen, B., Calvert, S., and Turpin, D., 1998, Nitrogen isotope fractionation during the uptake and assimilation of nitrate, nitrite, ammonium, and urea by a marine diatom: Limnology and Oceanography, v. 43, p. 215-224.

Weaver, P.F., Wall, J.D., and Gest, H., 1975, Characterization of *Rhodopseudomonas capsulata*: Archives of Microbiology, v. 105, p. 207-216.

Wellman, R.P., Cook, F.D., and Krouse, H.R., 1968, Nitrogen-15: microbiological alteration of abundance: Science (New York, N.Y.), v. 161, p. 269-270.

Wenk, C.B., Zopfi, J., Blees, J., Veronesi, M., Niemann, H., and Lehmann, M.F., 2014, Community N and O isotope fractionation by sulfide-dependent denitrification and anammox in a stratified lacustrine water column: Geochimica Et Cosmochimica Acta, v. 125, p. 551-563.

Wu, J., Calvert, S., and Wong, C., 1997, Nitrogen isotope variations in the subarctic northeast Pacific: relationships to nitrate utilization and trophic structure: Deep Sea Research Part I: Oceanographic Research Papers, v. 44, p. 287-314.

Wunderlich, A., Meckenstock, R.U., and Einsiedl, F., 2013, A mixture of nitrite-oxidizing and denitrifying microorganisms affects the δ 18 O of dissolved nitrate during anaerobic microbial denitrification depending on the δ 18 O of ambient water: Geochimica Et Cosmochimica Acta, v. 119, p. 31-45.

Wunderlich, A., Meckenstock, R., and Einsiedl, F., 2012, Effect of different carbon substrates on nitrate stable isotope fractionation during microbial denitrification: Environmental Science & Technology, v. 46, p. 4861-4868.