

Oxygen isotopic fractionation and exchange during bacterial nitrite oxidation

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Abstract

We elucidate the controls on the $\delta^{18}\text{O}$ values of microbially produced nitrate by tracking the $\delta^{18}\text{O}$ of nitrite and nitrate during bacterial nitrite oxidation, which is the final step of the nitrification process. Aside from the $\delta^{18}\text{O}$ values of the nitrite and water substrates, three factors can affect the $\delta^{18}\text{O}$ value of nitrate produced during nitrite oxidation: (1) a kinetic isotope effect for nitrite oxidation ($^{18}\epsilon_{k,\text{NO}_2}$), (2) a kinetic isotope effect for water incorporation by nitrite oxidoreductase ($^{18}\epsilon_{k,\text{H}_2\text{O},2}$), and (3) microbially mediated exchange of oxygen atoms between nitrite and water (x_{NOB}). These parameters were quantified through batch culture experiments with species from three genera of marine nitrite-oxidizing bacteria: *Nitrococcus*, *Nitrobacter*, and *Nitrospira*. Experiments conducted with ^{18}O -labeled water showed that less than 3% of the oxygen atoms in nitrite were exchanged with water and that $^{18}\epsilon_{k,\text{H}_2\text{O},2}$ ranged from +12.8‰ to +18.2‰. With the use of these parameters and the previously measured values of the $\delta^{18}\text{O}$ of nitrite produced by ammonia-oxidizing bacteria, the $\delta^{18}\text{O}$ of newly produced nitrate in the ocean was estimated to fall between -8.3% to -0.7% , which is within the range necessary for balancing a deep ocean nitrate $\delta^{18}\text{O}$ budget.

Introduction

Isotopic constraints on the global nitrogen budget—Nitrate (NO_3^-) is a limiting nutrient in many areas of the ocean, and its supply from deep water in part controls the productivity and subsequent carbon export from the surface ocean (Eppley and Peterson 1979). Understanding the controls on the oceanic nitrate inventory is therefore critical because of the implications for carbon storage in the ocean. Sources of fixed (bioavailable) nitrogen to the ocean include biological nitrogen fixation, atmospheric deposition, and continental runoff; the major sinks of fixed nitrogen from the ocean include denitrification and anaerobic ammonia oxidation (anammox) in marine sediments and suboxic water columns, as well as burial of organic nitrogen (Brandes and Devol 2002; Gruber 2004). Despite efforts to determine the magnitude of these sources and sinks of fixed nitrogen, the oceanic budget is still poorly constrained.

Nitrogen isotopic analyses of nitrate ($\delta^{15}\text{N}_{\text{NO}_3}$ [‰ vs. air]) = $\{(^{15}\text{R}_{\text{NO}_3}/^{15}\text{R}_{\text{air}}) - 1\} \times 1000$, where $^{15}\text{R} = ^{15}\text{N}:^{14}\text{N}$ and air refers to the standard atmospheric N_2) have provided the basis of several efforts to understand the balance of fixed nitrogen sources and sinks (Brandes and Devol 2002; Deutsch et al. 2004). However, this approach predicts a net fixed nitrogen loss from the ocean of 100–270 Tg N yr⁻¹ (Codispoti et al. 2001; Brandes and Devol 2002; Deutsch et al. 2004), whereas other approaches predict a balanced budget (Gruber and Sarmiento 1997; Gruber 2004). It is important to note that even those studies that predict a balanced budget have uncertainty of $\sim 30\%$ in the total source and sink fluxes.

Difficulty in resolving the $\delta^{15}\text{N}$ budget has prompted the use of nitrate $\delta^{18}\text{O}$ values ($\delta^{18}\text{O}_{\text{NO}_3}$ [‰ vs. VSMOW]) =

$\{(^{18}\text{R}_{\text{NO}_3}/^{18}\text{R}_{\text{VSMOW}}) - 1\} \times 1000$, where $^{18}\text{R} = ^{18}\text{O}:^{16}\text{O}$ and VSMOW refers to the standard Vienna Standard Mean Ocean Water) to constrain the marine fixed nitrogen budget (Sigman et al. 2009). The $\delta^{18}\text{O}$ value of deep ocean nitrate is controlled by a different set of processes than the $\delta^{15}\text{N}$ value (Fig. 1). Oxygen atoms are removed from the nitrate pool during assimilation and denitrification and are added during nitrification (Fig. 1). The $\delta^{18}\text{O}$ of deep ocean nitrate is fairly constant at about +1.5‰ to +2.5‰ (Sigman et al. 2005, 2009; Casciotti et al. 2008). For the $\delta^{18}\text{O}$ of deep ocean nitrate to be maintained in steady state, the $\delta^{18}\text{O}$ signature of the nitrification source must balance the flux-weighted $\delta^{18}\text{O}$ value of loss fluxes. To use the deep ocean nitrate $\delta^{18}\text{O}$ values to determine the relative effects of competing processes on the nitrate pool, the isotope effects associated with each source and sink process must be known.

A kinetic isotope effect ($^{15}\epsilon_k$, $^{18}\epsilon_k$) describes the ratio of the rates at which the heavy (^{15}N , ^{18}O) vs. light (^{14}N , ^{16}O) isotopes react in a given process. For example, $^{15}\epsilon_k(\%) = (^{14}k: ^{15}k - 1) \times 1000$ and $^{18}\epsilon_k(\%) = (^{16}k: ^{18}k - 1) \times 1000$, where k is the first-order rate constant for reaction of each isotopic species, indicated by superscripts 14 and 15 for nitrogen isotopes and 16 and 18 for oxygen isotopic species. The reduction of nitrate during water column denitrification and assimilation fractionates nitrate nitrogen and oxygen isotopes equally, leaving behind nitrate that is enriched in both ^{15}N and ^{18}O (Granger et al. 2004, 2008).

Given that the kinetic isotope effects for denitrification are nearly identical for both ^{18}O and ^{15}N , we would expect $^{18}\epsilon_k$ for water column and sedimentary denitrification to be approximately +25‰ and 0‰, respectively. Lehmann et al. (2004) have also provided empirical evidence for a small $^{18}\epsilon_k$ for sedimentary denitrification. The N and O isotope effects from assimilation are also identical and estimated to be approximately +5‰ (Granger et al. 2004). Consumption

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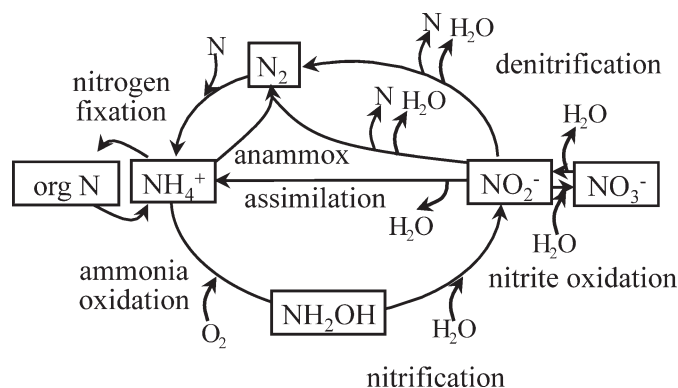


Fig. 1. Overview of microbial nitrogen cycle showing where nitrogen and oxygen atoms are added and removed from fixed nitrogen pools.

of nitrate by these processes should therefore increase the $\delta^{18}O$ value of the residual nitrate by removing nitrate with a low $\delta^{18}O$ value relative to average deep ocean values. When organic nitrogen is rematerialized, new oxygen atoms enter the nitrate pool through the process of nitrification (Fig. 1). Given that nitrate in the deep ocean has a $\delta^{18}O$ value of +1.5‰ to +2.5‰, the $\delta^{18}O$ of nitrate produced from nitrification must therefore be less than +1.5‰ to +2.5‰ to maintain $\delta^{18}O_{NO_3}$ in steady state.

Constraints on the $\delta^{18}O$ signature of newly produced nitrate—The established biochemical pathway for nitrification indicates that one oxygen atom comes from O_2 and the other two oxygen atoms come from water (Fig. 1; Andersson and Hooper 1983; Kumar et al. 1983; DiSpirito and Hooper 1986). If this simple stoichiometry determines the $\delta^{18}O$ value of nitrate produced by nitrification, then Eq. 1 can be used to calculate this value:

$$\delta^{18}O_{NO_3} = \frac{1}{3}(\delta^{18}O_{O_2}) + \frac{2}{3}(\delta^{18}O_{H_2O}) \quad (1)$$

With values of +23.5‰ to +36‰ for the $\delta^{18}O$ of O_2 ($\delta^{18}O_{O_2}$; Bender 1990) and 0‰ for the average $\delta^{18}O$ of seawater ($\delta^{18}O_{H_2O}$; Epstein and Mayeda 1953), the above equation predicts deep ocean nitrate $\delta^{18}O$ values of +7.8‰ to +12‰. Because this result is higher than the measured deep ocean nitrate value (+1.5‰ to +2.5‰), and given that consumption processes will further enrich the nitrate $\delta^{18}O$ value, factors beyond this simple stoichiometric relationship must come into play. We now know that isotope effects are associated with oxygen atom incorporation, as well as exchange of oxygen atoms between nitrite and water during ammonia oxidation that cause the $\delta^{18}O$ of newly produced nitrite (NO_2^-) to be lower than the stoichiometrically calculated value (Casciotti et al. 2010). In that study, it was determined that the nitrite produced through ammonia oxidation in seawater should have a $\delta^{18}O$ value of -3.3‰ to +5.3‰. The current study examines the hypothesis that there are additional isotope effects for nitrite oxidation to nitrate that might also lower the $\delta^{18}O$ of microbially produced nitrate relative to the simple stoichiometry commonly assumed (Eq. 1).

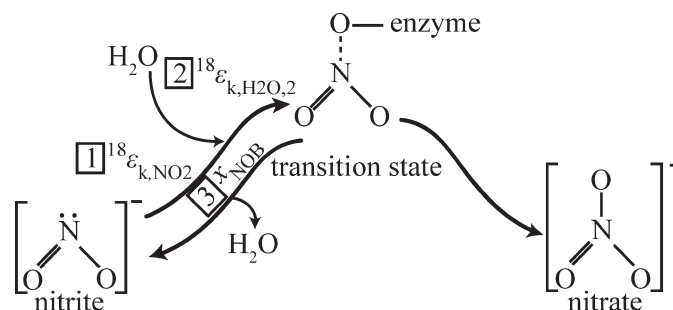


Fig. 2. Mechanism for nitrite oxidation, after Friedman et al. (1986), showing where isotope fractionation and exchange of oxygen occurs. The isotope effects associated with nitrite oxidation are labeled with numbers: (1) a kinetic isotope effect for nitrite oxidation ($^{18}\epsilon_{k,NO_2}$), (2) a kinetic isotope effect for water incorporation ($^{18}\epsilon_{k,H_2O,2}$), and (3) exchange between oxygen atoms in nitrite and water (x_{NOB}).

Biochemistry of nitrite oxidation—During nitrite oxidation, the enzyme nitrite oxidoreductase extracts an oxygen atom from water and then binds nitrite, forming an intermediate, or transition state (Friedman et al. 1986). The intermediate then either completes the oxidation to nitrate or back-reacts to nitrite by losing an oxygen atom (Fig. 2). During this process, three steps can affect the resulting $\delta^{18}O$ value in nitrate: (1) a kinetic isotope effect for nitrite oxidation ($^{18}\epsilon_{k,NO_2}$), (2) a kinetic isotope effect for water incorporation ($^{18}\epsilon_{k,H_2O,2}$; the subscript 2 represents the second water incorporated during nitrification, with the first occurring during ammonia oxidation), and (3) exchange of oxygen atoms between nitrite and water (x_{NOB} = the fraction of nitrite oxygen atoms that have been exchanged with water atoms before conversion to nitrate). $^{18}\epsilon_{k,NO_2}$ should only play a role in setting $\delta^{18}O_{NO_3}$ in situations in which nitrite utilization is incomplete, such as in water column primary or secondary nitrite maxima. $^{18}\epsilon_{k,H_2O,2}$ determines the $\delta^{18}O$ value at which the new oxygen atom from water is added. When this isotope effect is positive or “normal,” $H_2^{16}O$ is preferentially added to the nitrite over $H_2^{18}O$. Exchange of oxygen atoms between nitrite and water (x_{NOB}) occurs when the decomposition of the transition state results in loss of an oxygen atom from the original nitrite and retention of the oxygen atom from water (Fig. 2), which may also occur with an isotope effect (see below).

This study presents experiments in which the isotope effects and oxygen atom exchange associated with bacterially-mediated nitrite oxidation were measured. This is important because these values can be used to calculate an expected $\delta^{18}O$ value for the nitrate produced from nitrification, which can then be used to interpret the distribution of $\delta^{18}O_{NO_3}$ in the ocean and to create a fixed nitrogen budget using oxygen isotopes.

Methods

Maintenance of bacteria—All bacterial cultures used in this study were kindly provided by John Waterbury and Frederica Valois. *Nitrococcus mobilis* was originally isolated from the Pacific Ocean off the Galapagos Archipelago

Table 1. Summary of nitrite oxidation experiments.

Experiment	Bacterial species	$\delta^{18}\text{O}_{\text{H}_2\text{O}}$ in media (‰ vs. VSMOW)	x_{NOB} measured	$^{18}\epsilon_{\text{k,H}_2\text{O}_2}$ measured	$^{18}\epsilon_{\text{k,NO}_2}$ measured
1	<i>Nitrobacter</i> sp. Nb 355	-5.6	No	No	Yes
2	<i>Nitrospira marina</i>	-4.8	No	No	Yes
3	<i>Nitrococcus mobilis</i>	-6.2, +41.5	Yes	Yes	Yes
4	<i>Nitrococcus mobilis</i>	-5.5, +88.4	Yes	Yes	Yes
5	<i>Nitrobacter</i> sp. Nb 355	-5.2, +88.6	Yes	Yes	Yes
6	<i>Nitrococcus mobilis</i>	-5.5, +33.4, +73.4, +148.9	Yes	Yes	No
	<i>Nitrobacter</i> sp. Nb 355	-5.5, +33.4, +73.4, +148.9	Yes	Yes	No
7	<i>Nitrobacter</i> sp. Nb 297	-5.5, +33.4, +73.4, +148.9	Yes	Yes	No
	<i>Nitrococcus mobilis</i>	-5.1, +32.5, +72.6, +149.7	Yes	Yes	No
	<i>Nitrobacter</i> sp. Nb 355	-5.1, +32.5, +72.6, +149.7	Yes	Yes	No
	<i>Nitrobacter</i> sp. Nb 297	-5.1, +32.5, +72.6, +149.7	Yes	Yes	No

(Watson and Waterbury 1971). *Nitrobacter* sp. Nb 297 and *Nitrospira marina* were both originally isolated from the Gulf of Maine. Lastly, *Nitrobacter* sp. Nb 355 was isolated from Black Sea surface water. All bacteria were grown in a defined medium containing 75% artificial seawater and 25% distilled water amended with 400 $\mu\text{mol L}^{-1}$ MgSO_4 , 30 $\mu\text{mol L}^{-1}$ CaCl_2 , 5 $\mu\text{mol L}^{-1}$ KHPO_4 , 2.3 $\mu\text{mol L}^{-1}$ Fe(III)-EDTA ("Geigy iron"), 0.1 $\mu\text{mol L}^{-1}$ Na_2MoO_4 , 0.25 $\mu\text{mol L}^{-1}$ MnCl_2 , 0.002 $\mu\text{mol L}^{-1}$ CoCl_2 , and 0.08 $\mu\text{mol L}^{-1}$ ZnSO_4 (Watson and Waterbury 1971). Filter-sterilized NaNO_2 working stock (5 mol L^{-1}) was added after autoclaving to achieve 10 mmol L^{-1} NO_2^- in *Nitrococcus* and *Nitrobacter* cultures and 2 mmol L^{-1} in *Nitrospira* cultures. Media were adjusted to pH 8.2 with sterile K_2CO_3 after NO_2^- addition. All cultures were maintained in a 23°C dark incubator in 1-liter flasks.

Nitrite oxidation experiments—To measure the three different isotope effects during nitrite oxidation (Fig. 2), batch culture incubations were conducted with pure cultures of nitrite-oxidizing bacteria. Results from seven different experiments are reported here. The experiments differed slightly on the basis of the bacterium used, the media $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ values, and the sampling frequency. These details are summarized in Table 1.

Media for incubation experiments were prepared as described above, with the following modifications. First, ^{18}O -labeled water (with a $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ value of approximately +5000‰ vs. VSMOW) was added in amounts of 3–33 mL L^{-1} of medium to achieve $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ values for the media of -5‰ to +150‰ vs. VSMOW. The labeled water was added before autoclaving to ensure adequate mixing of the labeled water throughout the media. After autoclaving, NaNO_2 was added to 50 $\mu\text{mol L}^{-1}$, and the media were neutralized to pH 8.2 with sterile K_2CO_3 .

The maintenance cultures (300–500 mL) were harvested either by centrifugation (4000 rpm for 30 min) or filtration (0.22- μm pore size filter), depending on the cell density in the culture. The harvested bacteria were then washed and resuspended with 0.22- μm filtered artificial seawater. The experiments were initiated with inoculation of the bacteria into the prepared media. In experiments in which $^{18}\epsilon_{\text{k,NO}_2}$ was measured, duplicate flasks were inoculated

for each initial $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ value. One flask received twice the amount of inoculum as the other flask to gain higher resolution for the isotope effect estimate over the full range of nitrite consumption. In all other experiments, replicate incubations received the same amount of inoculum. In all experiments, sterile controls were analyzed in parallel to check for abiotic oxidation of nitrite to nitrate.

After inoculation, the flasks were subsampled (10–15 mL) immediately and then periodically throughout conversion of nitrite to nitrate and again after all nitrite had been consumed. Each subsample was 0.22- μm filtered immediately after collection. Nitrite concentrations were measured immediately after sampling and then used to calculate the sample volume needed to obtain 5–20 nmol of nitrite for isotopic analysis. Azide reactions for nitrite isotope analyses (see below for details) were conducted within 1 h of sampling to avoid abiotic exchange of oxygen atoms between nitrite and water during storage of samples (Casciotti et al. 2007). The rest of each subsample was stored frozen in 15-mL centrifuge tubes until analyses for nitrate concentration and isotopic composition.

Concentration analyses—Nitrite concentrations were analyzed according to the Greiss–Ilosvay colorimetric reaction (Strickland and Parsons 1972). Samples were reacted in duplicate and measured at a wavelength of 543 nm on Amersham Biosciences Ultrospec 2100 spectrophotometer with a Gilson 220XL autosampler. Nitrate plus nitrite concentrations were analyzed in duplicate following the chemiluminescence detection method after hot vanadium (III) reduction to nitric oxide (Braman and Hendrix 1989). Samples (1 mL) were injected into a flask containing vanadium chloride held at 95°C, and the resulting nitric oxide was measured in a Nitrogen Oxides Detector (model 8840, Monitor Labs) against nitrate concentration standards prepared gravimetrically.

Isotopic analyses—Nitrite $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values were measured with the azide method developed by McIlvin and Altabet (2005). Briefly, sample nitrite was reduced to nitrous oxide in a sealed 20-mL vial with 2 mol L^{-1} sodium azide in 20% acetic acid. The nitrous oxide analyte was

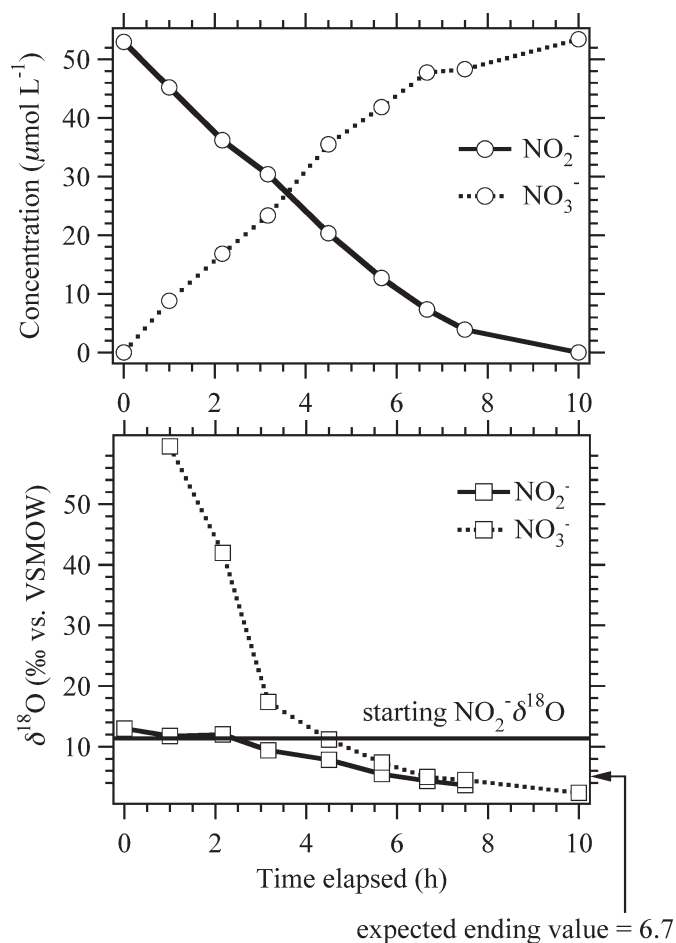


Fig. 3. (a) Nitrite and nitrate concentrations and (b) $\delta^{18}\text{O}$ values of nitrite and nitrate during a typical time course incubation with *Nitrococcus mobilis* (Expt. 3). The $\delta^{18}\text{O}_{\text{NO}_2}$ started at a value of +13‰ and decreased over time because of the (inverse) isotope effect for nitrite oxidation ($^{18}\epsilon_{\text{k,NO}_2}$). The $\delta^{18}\text{O}_{\text{NO}_3}$ of produced nitrate is initially high and then decreases over time because of both closed-system mass balance constraints and the incorporation of H_2O with a low $\delta^{18}\text{O}$ value. Eventually, $\delta^{18}\text{O}_{\text{NO}_3}$ reaches a value that reflects the complete oxidation of NO_2^- to NO_3^- and the incorporation of H_2O with a kinetic isotope effect ($^{18}\epsilon_{\text{k,H}_2\text{O},2}$).

then purged from the sample vial, trapped cryogenically with liquid nitrogen, and measured for nitrogen and oxygen isotopic composition on a Finnigan Delta^{PLUS} XP isotope ratio mass spectrometer. The volume of sample analyzed was calculated from the concentration of nitrite to obtain 5–20 nmol of N. Nitrite isotopic standards N23, N7373, and N10219— NaNO_2 salts with known $\delta^{18}\text{O}_{\text{NO}_2}$ values of +4.5‰, +11.4‰, and +88.5‰, respectively (Casciotti et al. 2007)—were analyzed in parallel, with amounts tuned to match the samples (5–20 nmol N). The maximum sample volume that could be used in the isotopic analysis was 10 mL, meaning at least $0.5 \mu\text{mol L}^{-1}$ nitrite needed to be present to obtain an accurate isotopic measurement with our system. Precision on replicate $\delta^{18}\text{O}_{\text{NO}_2}$ analyses is 0.5% (McIlvin and Altabet 2005).

Nitrate (plus nitrite) $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ analyses were made by the denitrifier method (Sigman et al. 2001; Casciotti et al. 2002). The sample volumes were calculated with the combined nitrite and nitrate concentrations to obtain 5–20 nmol of nitrite plus nitrate-N in each vial. Both nitrite and nitrate standards were analyzed with each denitrifier run to allow mass and isotope subtraction of nitrite from the combined (nitrate plus nitrite) isotope measurements (Casciotti and McIlvin 2007). The nitrate standards used were USGS32, USGS34, and USGS35, with known $\delta^{18}\text{O}_{\text{NO}_3}$ values of +25.7‰, –27.9‰, and +57.5‰, respectively (Böhlke et al. 2003). The same nitrite standards employed in the azide method were reanalyzed by the denitrifier method to calibrate the nitrite isotopic contribution to samples containing both nitrate and nitrite (Casciotti and McIlvin 2007). Samples containing both nitrite and nitrate were measured using both azide and denitrifier methods, and the nitrate isotopes were calculated using a mass balance subtraction described in Casciotti and McIlvin (2007). Precision on replicate $\delta^{18}\text{O}_{\text{NO}_3}$ analyses is approximately 0.6% (Casciotti and McIlvin 2007).

The $\delta^{18}\text{O}$ of water was measured according to the method described in McIlvin and Casciotti (2006), which uses rapid abiotic oxygen isotope exchange between nitrite and water at low pH (Bunton et al. 1959), followed by the azide method for nitrite $\delta^{18}\text{O}$ analysis. Water samples received $5 \mu\text{L}$ of 6 mol L^{-1} hydrochloric acid (HCl) per 0.4-mL sample to bring the pH below 4. Samples containing no nitrite were amended with 5–20 nmol nitrite before acidification, whereas samples containing nitrite did not require this addition. The addition of the acid facilitates abiotic equilibration of oxygen atoms between nitrite and water, so that the equilibrated $\delta^{18}\text{O}$ value of the nitrite reflects that of the water plus an equilibration isotope effect of approximately +14‰ at room temperature (Casciotti et al. 2007). After equilibration, the azide method for nitrite isotopes was conducted following the above procedure. Sample analyses were calibrated against water standards $\text{H}_2\text{O}-1$, $\text{H}_2\text{O}-2$, and $\text{H}_2\text{O}-3$, with independently calibrated $\delta^{18}\text{O}$ values of –5.82‰, +18.04‰, +41.35‰, respectively, vs. VSMOW (McIlvin and Casciotti 2006). The water standards were run in triplicate, with each set of water samples receiving the same nitrite, HCl, and azide treatments. Precision of replicate $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ analyses is 0.5% (McIlvin and Casciotti 2006).

Results

Oxidation of nitrite to nitrate was complete in all experiments. Experiments with *Nitrococcus* and *Nitrobacter*, which grew to higher densities ($\sim 10^7$ cells L^{-1}) completely consumed the nitrite in 10–48 h. The *Nitrospira* cultures, which grew to a lower cell density, took 2 weeks to consume all of the nitrite. Figure 3 shows the nitrate and nitrite concentrations and $\delta^{18}\text{O}$ values for a typical time course incubation of *N. mobilis* in medium with a $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ value of –6‰. In this experiment, $\delta^{18}\text{O}_{\text{NO}_2}$ decreased over time as it was oxidized to nitrate. $\delta^{18}\text{O}_{\text{NO}_3}$ was initially elevated relative to nitrite but obtained a value of –0.8‰ once all of the NO_2^- had been oxidized to NO_3^- . These

results reflect the combination of isotope effects for nitrite oxidation ($^{18}\epsilon_{k,NO_2}$) and water incorporation ($^{18}\epsilon_{k,H_2O,2}$), as will be discussed in detail below. However, we emphasize here that the final $\delta^{18}O$ value of the nitrate (-0.8%) was lower than expected ($+6.7\%$) on the basis of Eq. 2, which neglects any isotope effects.

$$\delta^{18}O_{NO_3 \text{ final}} = \frac{2}{3}(\delta^{18}O_{NO_2 \text{ initial}}) + \frac{1}{3}(\delta^{18}O_{H_2O}) \quad (2)$$

The deviation of the $\delta^{18}O_{NO_3 \text{ final}}$ from Eq. 2 could be the result of isotope effects associated with either water incorporation ($^{18}\epsilon_{k,H_2O,2}$) or the exchange of oxygen between nitrite and water (x_{NOB}). The isotope effect for nitrite selection ($^{18}\epsilon_{k,NO_2}$) can be neglected in this case because nitrite was eventually consumed fully. We can amend Eq. 2 to include these factors:

$$\begin{aligned} \delta^{18}O_{NO_3 \text{ final}} = & \frac{2}{3} [(1 - x_{NOB})\delta^{18}O_{NO_2 \text{ initial}} \\ & + x_{NOB}(\delta^{18}O_{H_2O} + ^{18}\epsilon_{eq})] \\ & + \frac{1}{3}(\delta^{18}O_{H_2O} - ^{18}\epsilon_{k,H_2O,2}) \end{aligned} \quad (3)$$

As defined previously, x_{NOB} is the fraction of nitrite oxygen atoms that have exchanged with water before conversion to nitrate. A nonzero value for x_{NOB} causes the $\delta^{18}O$ of the reacting nitrite to change over time, which introduces an additional dependence on the $\delta^{18}O$ of water in Eq. 3. During equilibration, there is also an equilibrium isotope effect ($^{18}\epsilon_{eq}$), which causes the water that “sticks” to nitrite to have a higher $\delta^{18}O$ value than the bulk water.

We can determine values for x_{NOB} and $^{18}\epsilon_{k,H_2O,2}$ by analyzing data from parallel incubations conducted at a variety of $\delta^{18}O_{H_2O}$ values. This becomes apparent after rearranging Eq. 3 with $\delta^{18}O_{H_2O}$ as the independent variable:

$$\begin{aligned} \delta^{18}O_{NO_3 \text{ final}} = & \left[\frac{2}{3}(x_{NOB}) + \frac{1}{3} \right] \delta^{18}O_{H_2O} \\ & + \left\{ \frac{2}{3} [(1 - x_{NOB})\delta^{18}O_{NO_2 \text{ initial}} \right. \\ & \left. + ^{18}\epsilon_{eq}(x_{NOB})] - \frac{1}{3} ^{18}\epsilon_{k,H_2O,2} \right\} \end{aligned} \quad (4)$$

If x_{NOB} and $^{18}\epsilon_{k,H_2O,2}$ are constant for a given experiment (do not change with different $\delta^{18}O_{H_2O}$ values), then the slope (m) and intercept (b) of this linear equation are constants. Therefore, if $\delta^{18}O_{NO_2 \text{ initial}}$, $^{18}\epsilon_{eq}$, and $\delta^{18}O_{NO_3 \text{ final}}$ are known, then x_{NOB} can be calculated from the slope and $^{18}\epsilon_{k,H_2O,2}$ from the intercept of Eq. 4.

Nitrite and water exchange (x_{NOB})— $\delta^{18}O_{NO_3 \text{ final}}$ values corresponding to the media $\delta^{18}O_{H_2O}$ values of five experiments are plotted in Fig. 4. The slopes from the linear regressions for each experiment and bacterial species were similar to 0.333 and did not show species-dependent variations (Table 2). The slopes of the linear regressions

from Fig. 4 can then be used to calculate x_{NOB} values from the coefficient of $\delta^{18}O_{H_2O}$ term from Eq. 4:

$$m = \frac{2}{3}(x_{NOB}) + \frac{1}{3} \quad (5)$$

These x_{NOB} values have been calculated (Table 2) by solving Eq. 5 individually for each bacterial species and experiment. The uncertainty in x_{NOB} for each experiment was calculated from the uncertainty of the linear fit. All values of x_{NOB} were less than 3%, indicating that there was little exchange between nitrite and water during nitrite oxidation. The average and standard deviation of x_{NOB} in all experiments using *N. mobilis*, *Nitrobacter* sp. Nb 355, and *Nitrobacter* sp. Nb 297 were $1.5\% \pm 0.8\%$, $0.2\% \pm 0.9\%$, and $0.3\% \pm 0.5\%$, respectively. The differences between species were not statistically significant ($p > 0.05$).

Water incorporation isotope effect ($^{18}\epsilon_{k,H_2O,2}$)—Whereas the slopes of the linear regression of $\delta^{18}O_{H_2O}$ vs. $\delta^{18}O_{NO_3 \text{ final}}$ were all very similar between bacteria and experiments, the intercepts showed more variability (Fig. 4; Table 2). The equation that describes the intercept is:

$$\begin{aligned} b = & \frac{2}{3} [(1 - x_{NOB})\delta^{18}O_{NO_2 \text{ initial}} + ^{18}\epsilon_{eq}(x_{NOB})] \\ & - \frac{1}{3} ^{18}\epsilon_{k,H_2O,2} \end{aligned} \quad (6)$$

The kinetic isotope effect for water incorporation ($^{18}\epsilon_{k,H_2O,2}$) can be calculated by rearranging Eq. 6 and substituting in measured values of b, x_{NOB} , and $\delta^{18}O_{NO_2 \text{ initial}}$ and an estimate of $^{18}\epsilon_{eq}$.

$$\begin{aligned} ^{18}\epsilon_{k,H_2O,2} = & -3b + 2 \\ & \left[\left(\frac{3}{2} - \frac{3}{2}m \right) \delta^{18}O_{NO_2 \text{ initial}} + ^{18}\epsilon_{eq} \left(\frac{3}{2}m - \frac{1}{2} \right) \right] \end{aligned} \quad (7)$$

A value of $+14\%$ for $^{18}\epsilon_{eq}$, as measured by Casciotti et al. (2007) for abiotic nitrite and water oxygen isotope exchange, was used here to calculate $^{18}\epsilon_{k,H_2O,2}$. We justify this assumption by noting that although enzymes might accelerate the approach to equilibrium, they are not expected to alter the equilibrium point or the isotope effect between two species at equilibrium. Moreover, given the low amount of exchange estimated for nitrite oxidation (Table 2) our estimates of $^{18}\epsilon_{k,H_2O,2}$ are not very sensitive to this assumption, which affects estimates of $^{18}\epsilon_{k,H_2O,2}$ by only 0.05% for every 1% change in $^{18}\epsilon_{eq}$. The averages and standard deviations of $^{18}\epsilon_{k,H_2O,2}$ across experiments were $+17.8\% \pm 4.7\%$, $+12.3\% \pm 3.0\%$, and $+15.6\% \pm 0.03\%$ for *N. mobilis*, *Nitrobacter* sp. Nb 355, and *Nitrobacter* sp. Nb 297, respectively (Table 2). The greater variability in the *N. mobilis* and *Nitrobacter* sp. Nb 355 can be attributed to results from Expts. 3 and 5, in which the intercepts were anomalously low and high, respectively. If these outliers are discarded, the isotope effects for water incorporation would be $15.4\% \pm 0.3\%$ and $14.0\% \pm 0.9\%$ for *N. mobilis* and *Nitrobacter* sp. Nb 355, respectively, which are much closer to the isotope effect for *Nitrobacter* sp. Nb 297.

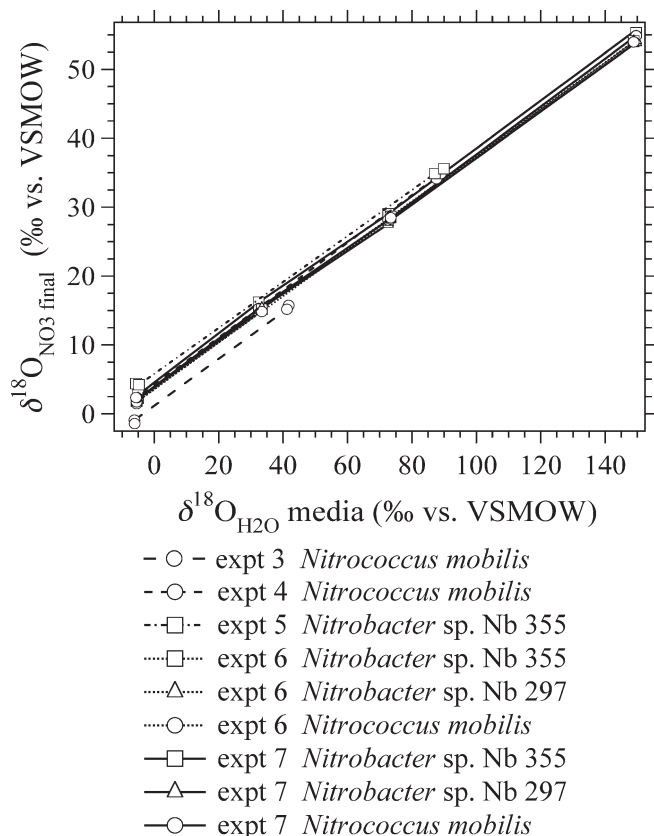


Fig. 4. $\delta^{18}\text{O}_{\text{NO}_3}$ produced in different $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ media after all NO_2^- has been oxidized to NO_3^- in Expts. 3, 4, 5, 6, and 7. Slopes and intercepts of the linear regressions were used to calculate x_{NOB} and $^{18}\epsilon_{\text{k,H}_2\text{O},2}$, as shown in Table 2.

However, in Expts. 6 and 7, in which the intercepts and derived $^{18}\epsilon_{\text{k,H}_2\text{O},2}$ values were more consistent within and between species, *Nitrobacter* sp. Nb 355 did display a statistically lower $^{18}\epsilon_{\text{k,H}_2\text{O},2}$ than the other two species within a 90% confidence interval ($p = 0.08$). In each case, the measured $^{18}\epsilon_{\text{k,H}_2\text{O},2}$ is positive or “normal,” meaning that the $\delta^{18}\text{O}$ value of water molecules incorporated into nitrate is lower than the $\delta^{18}\text{O}$ value of the bulk water, indicating that nitrite-oxidizing bacteria are preferentially incorporating H_2^{16}O into nitrate.

Nitrite kinetic isotope effect ($^{18}\epsilon_{\text{k,NO}_2}$)—Figure 5 shows the $\delta^{18}\text{O}$ of nitrite plotted vs. $-\ln(f)$, where f is the fraction of nitrite remaining ($f = [\text{NO}_2^-]/[\text{NO}_2^-]_{\text{initial}}$), for the five experiments in which $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ was approximately -6‰ . In closed-system Rayleigh fractionation with a constant kinetic isotope effect, a linear relationship is expected between $-\ln(f)$ and $\delta^{18}\text{O}_{\text{NO}_2}$, with the slope of the regression equaling $^{18}\epsilon_{\text{k,NO}_2}$ (Mariotti et al. 1981; Scott et al. 2004). However, the data from our experiments were found to fit a quadratic function more closely, implying that the isotope effect decreased linearly over the course of the experiment (Fig. 5).

Oxygen atom exchange could cause curvature in the Rayleigh plots for $\delta^{18}\text{O}_{\text{NO}_2}$, depending on the relative $\delta^{18}\text{O}$ values of NO_2^- and H_2O . In fact, we believe that exchange did cause curvature in experiments conducted with higher $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ media, where isotopic fractionation (decreasing $\delta^{18}\text{O}_{\text{NO}_2}$) and exchange (increasing $\delta^{18}\text{O}_{\text{NO}_2}$) produced counteracting effects. However, in experiments conducted in the media with the lowest $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ value (-6‰ ; Fig. 5), exchange with an equilibrium isotope effect of $+14\text{‰}$ would push $\delta^{18}\text{O}_{\text{NO}_2}$ toward $+8\text{‰}$. At the lowest $\delta^{18}\text{O}$ values reached ($+1\text{‰}$; Fig. 5), 3% exchange would cause a change of only 0.4‰ in $\delta^{18}\text{O}_{\text{NO}_3}$. Therefore, in the experiments presented in Fig. 5, we can exclude exchange as a factor in producing the observed curvature.

As the experiments progressed, the concentrations of nitrite would also have been decreasing, suggesting that the magnitude of the expressed isotope effect might be dependent on nitrite concentration. We have tested this by conducting additional experiments with *N. mobilis* at four different initial concentrations of nitrite, ranging from 5 to $500 \mu\text{mol L}^{-1}$. The expressed kinetic isotope effects for nitrite oxidation ($^{15}\epsilon_{\text{k,NO}_2}$ and $^{18}\epsilon_{\text{k,NO}_2}$ for ^{15}N and ^{18}O , respectively) were smaller at lower initial nitrite concentrations (not shown). This is consistent with concentration dependence underlying the quadratic fit, yielding the decreasing slopes near the end of the experiments presented in Fig. 5. Similar patterns have been observed in other systems and have been interpreted to reflect the effects of co-occurring processes, such as transport and reaction of substrate (Farquhar et al. 1982; Needoba et al. 2004). In these cases, it was shown that the enzyme-level kinetic isotope effect is expressed more strongly at high concen-

Table 2. Results for water incorporation ($^{18}\epsilon_{\text{k,H}_2\text{O},2}$) and exchange (x_{NOB}).

Bacteria	Expt.	Slope (m)	Intercept (b)	x_{NOB} (fraction)	$^{18}\epsilon_{\text{k,H}_2\text{O},2}$ (‰)
<i>Nitrococcus mobilis</i>	3	0.351 ± 0.006	0.8 ± 0.2	0.026 ± 0.006	24.9 ± 0.9
	4	0.343 ± 0.001	4.2 ± 0.1	0.015 ± 0.001	15.0 ± 1.6
	6	0.339 ± 0.001	3.5 ± 0.0	0.008 ± 0.001	15.6 ± 0.5
	7	0.341 ± 0.002	3.7 ± 0.1	0.011 ± 0.002	15.6 ± 1.6
	Average			0.015 ± 0.008	17.8 ± 4.7
<i>Nitrobacter</i> sp. Nb 355	5	0.328 ± 0.002	6.1 ± 0.2	-0.008 ± 0.002	9.0 ± 1.0
	6	0.337 ± 0.000	3.9 ± 0.0	0.006 ± 0.000	14.6 ± 0.5
	7	0.339 ± 0.002	4.5 ± 0.1	0.009 ± 0.002	13.3 ± 1.6
	Average			0.002 ± 0.009	12.3 ± 3.0
<i>Nitrobacter</i> sp. Nb 297	6	0.338 ± 0.001	3.6 ± 0.1	0.007 ± 0.001	15.6 ± 0.5
	7	0.333 ± 0.002	3.7 ± 0.2	0.000 ± 0.002	15.7 ± 1.6
	Average			0.003 ± 0.005	15.6 ± 0.03

trations of substrate, gradually approaching the (smaller) isotope effect for transport at low substrate concentrations. Our results are consistent with a similar concentration-dependent expression of the isotope effect for nitrite oxidation.

To compare the most relevant enzyme-level kinetic isotope effects between experiments, $^{18}\epsilon_{k,NO_2}$ was calculated at time 0 by using the linear coefficient of the quadratic fit in experiments that started with $50 \mu\text{mol L}^{-1}$ nitrite (Table 3). *N. mobilis* and *Nitrobacter* sp. Nb 355 had $^{18}\epsilon_{k,NO_2}$ values averaging $-8.2\% \pm 2.5\%$ and $-6.5\% \pm 1.8\%$, respectively, which are not significantly different ($p > 0.05$). *N. marina*, however, had a significantly lower $^{18}\epsilon_{k,NO_2}$ value of $-1.3\% \pm 0.4\%$ compared with the other two species ($p < 0.05$).

The observed $^{18}\epsilon_{k,NO_2}$ values were negative or “inverse” for all experiments, indicating that the nitrite became depleted in the heavy isotope (^{18}O) as the pool was consumed. This might be surprising at first, given that most enzymatic reactions cause heavy-isotope enrichment in the substrate pool. However, it has previously been shown that an inverse isotope effect is to be expected for N-isotope fractionation in this simple bond-forming reaction (Casciotti 2009). Here we observe inverse kinetic fractionation for both N and O isotopes, with the ^{18}O isotope effects being smaller, or less inverse, than the ^{15}N isotope effects (Table 3).

Discussion

Reaction mechanism and oxygen isotope exchange (χ_{NOB})—During nitrite oxidation, the enzyme nitrite oxidoreductase first binds to an oxygen atom from water, forming an enzyme–oxygen complex. This complex then binds nitrite to form an enzyme-bound intermediate that can either decompose back to nitrite or proceed to form nitrate (Friedman et al. 1986; Fig. 2). If the intermediate loses one of the original nitrite oxygen atoms during back-reaction, some of the oxygen atoms in the original nitrite will have been replaced by water. Such an exchange would be expected to alter the $\delta^{18}\text{O}$ of nitrite over time and lead to an increased dependence of nitrite and nitrate $\delta^{18}\text{O}$ on variations in water $\delta^{18}\text{O}$, which was not observed in our experiments. Our observations are consistent with earlier studies, which also showed little, if any, exchange between nitrite and water during nitrite oxidation (DiSpirito and Hooper 1986; Friedman et al. 1986). This implies that either back-reaction within the nitrite oxidoreductase enzyme is minimal or that it routinely extracts the oxygen atom added from H_2O .

During back-reaction from the transition state, the removal of the new oxygen atom would require breaking only one N–O bond, whereas removal of one of the original nitrite oxygen atoms would require breaking two N–O bonds and reforming one. Less energy would be required to break the one bond (between the water oxygen and nitrite nitrogen) than between two bonds (between the water oxygen and the enzyme and between the nitrite nitrogen and oxygen), lending some support for preferential removal of the oxygen atom added from H_2O . Also, because of

resonance within the double-bond structure of the original nitrite molecule, these bonds are most likely stronger than the newly formed bond and thus harder to break. Therefore, the defined mechanism for the addition of oxygen during nitrite oxidation supports our observations of little isotopic exchange catalyzed between nitrite and water during nitrite oxidation, and it is unlikely that this will change under different growth conditions or with other species.

Inverse kinetic isotope effect ($^{18}\epsilon_{k,NO_2}$)—An inverse isotope effect is rare in biology but could be expected for this bond-forming reaction because of stabilization of the transition state by the heavier isotopes (Casciotti 2009). An inverse isotope effect for nitrite oxidation in *N. mobilis* has been observed previously for ^{15}N ($^{15}\epsilon_{k,NO_2} \approx -13\%$; Casciotti 2009). In the current study, we observed inverse kinetic isotope effects for both oxygen isotope fractionation ($^{18}\epsilon_{k,NO_2}$) and nitrogen isotope fractionation ($^{15}\epsilon_{k,NO_2}$) during nitrite oxidation (Table 3). The observed $^{18}\epsilon_{k,NO_2}$ values were smaller in magnitude than the $^{15}\epsilon_{k,NO_2}$ values in all cases, perhaps because the nitrite oxygen atoms are not directly involved with formation of the new bond, whereas the nitrogen atom is. Heavy isotope substitution for oxygen might be expected to lower the vibrational frequencies of the nitrite molecule overall but are expected to have a smaller, secondary effect on the oxidation rates of nitrite to nitrate.

The magnitude of isotopic fractionation was similar in *N. mobilis* and *Nitrobacter* species but significantly smaller in *N. marina*. These differences could be related to the location of nitrite oxidation (cytoplasm vs. periplasm), as discussed below for $^{18}\epsilon_{k,H_2O,2}$, or differences in the enzyme active sites of the nitrite-oxidizing systems of these organisms. Substrate concentration dependence, which was observed to differing extents in our experiments (less in *Nitrospira* than in *Nitrobacter* or *Nitrococcus*), could also be exacerbated by the location of the nitrite-oxidizing system and the dependence on cellular transport of nitrite before oxidation. It has been shown in other systems that the concentration of substrate (nitrite, in this case) can have an effect on the expressed isotope effect because of transport limitation (Farquhar et al. 1982; Granger et al. 2004; Needoba et al. 2004). If lower nitrite concentrations cause diffusive limitation, there would be less expression of the enzyme isotope effect, and the transport isotope effect (which is commonly small and positive) would be expressed instead. Further experimentation to manipulate these experimental variables might reveal systematic changes in the $\delta^{18}\text{O}$ isotope effects for nitrite oxidation.

Interpretations of $\Delta(15,18)$ —In the introduction of the nitrate isotope anomaly, $\Delta(15,18)$, Sigman et al. (2005) reasoned that this anomaly could be caused by two processes occurring in suboxic water columns: remineralization of newly fixed nitrogen and nitrite reoxidation. In modeling the contributions of these processes, the best available information about the oxygen isotope systematics of nitrification were used to assign a $\delta^{18}\text{O}$ of 0% for NO_3^- produced via nitrification and nitrite reoxidation. This was

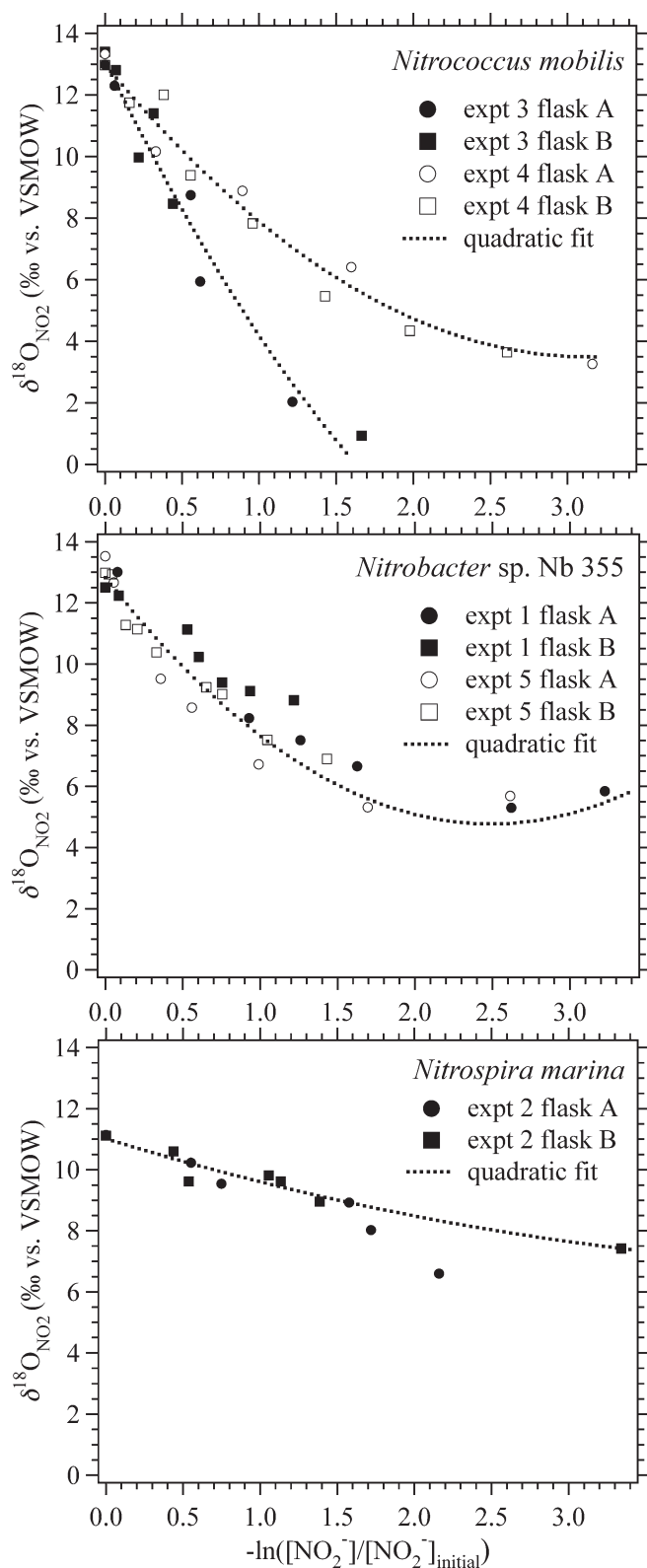


Fig. 5. Rayleigh plots for nitrite oxidation for three different species of bacteria in five different experiments. In panel A, quadratic fits are shown independently for experiments 3 and 4. In panel B, data are combined from experiments 1 and 5 to produce the fit. In panel C, the quadratic fit is shown for experiment 2. Derived values for $^{18}\epsilon_{k,NO_2}$ are given in Table 3.

based on the assumptions that the produced nitrate reflects complete exchange of oxygen isotopes between nitrite and water (with no equilibrium isotope effect) followed by the addition of water with no isotope effect. Casciotti and McIlvin (2007) used much the same model for determining the amounts of nitrite reoxidation, remineralized newly fixed nitrogen, or both, except with the incorporation of an equilibrium isotope effect for equilibration between nitrite and water of +14‰.

Since then, our experiments have provided further insight into the mechanisms of variation in $\delta^{18}O_{NO_3}$ and the corresponding $\Delta(15,18)$ values. Our current results suggest that $\delta^{18}O_{NO_3}$ of microbially produced nitrate could be much lower than 0‰ (see below). Using these lower $\delta^{18}O_{NO_3}$ values, it would be more difficult to produce the observed negative $\Delta(15,18)$ signals from either remineralization of newly fixed nitrogen or nitrite reoxidation. That is, a greater flux of nitrate from remineralization or reoxidation would be needed to generate the observed $\Delta(15,18)$ signals. Furthermore, interpretation of $\Delta(15,18)$ in the euphotic zone of Monterey Bay suggested that negative $\delta^{18}O$ values for microbially produced nitrate could even lead to positive $\Delta(15,18)$ anomalies in the euphotic zone, where nitrification supplies a large fraction of the available NO_3^- (Wankel et al. 2007).

On the other hand, the inverse kinetic isotope effect for nitrite oxidation could also be expressed in and around suboxic zones where nitrite accumulates up to $10 \mu\text{mol L}^{-1}$. In these regions, the observed $\delta^{18}O$ of nitrate is higher than the more constant deep ocean value, not only because of the fractionation imposed by the reduction of nitrate, but perhaps also from the reoxidation of nitrite to nitrate (Sigman et al. 2005; Casciotti and McIlvin 2007). An inverse isotope effect during nitrite oxidation would cause the $\delta^{18}O$ of nitrate to be higher than expected for a normal isotope effect, which has been previously assumed when interpreting $\Delta(15,18)$ values (Sigman et al. 2005; Casciotti and McIlvin 2007). In this case, an inverse kinetic isotope effect would make nitrite reoxidation more efficient at generating nitrate isotope anomalies, and perhaps less reoxidation would be required to explain a given $\Delta(15,18)$ anomaly.

Separating the effects of nitrite reoxidation and nitrogen fixation on $\Delta(15,18)$ in suboxic water columns remains a challenge, and additional lines of evidence are needed to determine the relative contribution of each process. We have proposed that the $\delta^{15}N$ difference between NO_2^- and NO_3^- ($\Delta\delta^{15}N = \delta^{15}N_{NO_3} - \delta^{15}N_{NO_2}$; Casciotti 2009) could provide an independent estimate of nitrite reoxidation. Nitrite oxygen isotope measurements might provide additional insight on this problem.

Implications for a global fixed nitrogen budget—The balance of oceanic fixed nitrogen inputs and outputs is currently poorly constrained. The isotopes of nitrogen and oxygen in deep-water nitrate can help constrain the sources and sinks of fixed nitrogen, although the best estimates of these fluxes are only possible with additional knowledge of isotope effects associated with each process. The $\delta^{18}O$ value of nitrate produced during nitrification is one important piece of information required to create a $\delta^{18}O$ budget for

Table 3. Measured $^{18}\epsilon_{k,NO_2}$ and $^{15}\epsilon_{k,NO_2}$ for three different species of nitrite-oxidizing bacteria.

Bacteria	Experiment	Flask	$^{18}\epsilon_{k,NO_2}$ (‰)	Average $^{18}\epsilon_{k,NO_2}$ (‰)	$^{15}\epsilon_{k,NO_2}$ (‰)	Average $^{15}\epsilon_{k,NO_2}$ (‰)
<i>Nitrococcus mobilis</i>	3	A	-10.2	-8.2 ± 2.5	-20.8	-20.2 ± 2.8
		B	-10.4		-22.0	
	4	A	-5.2	-6.5 ± 1.8	-16.0	-20.6 ± 3.2
		B	-7.0		-21.8	
<i>Nitrobacter</i> sp. Nb 355	1	A	-5.8	-6.5 ± 1.8	-18.1	-20.6 ± 3.2
		B	-4.4		-17.5	
	5	A	-8.7	-1.3 ± 0.4	-23.1	-9.1 ± 1.8
		B	-7.0		-23.6	
<i>Nitrospira marina</i>	2	A	-1.0	-1.3 ± 0.4	-7.8	-9.1 ± 1.8
		B	-1.5		-10.3	

oceanic nitrate and to interpret gradients in oceanic $\delta^{18}O_{NO_3}$ values.

From the results presented here, the value for $\delta^{18}O_{NO_3}$ produced by microbial nitrification in the ocean can now be estimated from Eq. 8, which is derived from Eq. 3 assuming $x_{NOB} = 0$.

$$\delta^{18}O_{NO_3 \text{ produced}} = \frac{2}{3} \delta^{18}O_{NO_2 \text{ source}} + \frac{1}{3} (\delta^{18}O_{H_2O} - ^{18}\epsilon_{k,H_2O,2}) \quad (8)$$

With the use of previously measured values for the $\delta^{18}O$ of nitrite produced by ammonia-oxidizing bacteria (-3.3% to $+5.3\%$; Casciotti et al. 2010), a $\delta^{18}O_{H_2O}$ value equal to 0% for seawater, and the $^{18}\epsilon_{k,H_2O,2}$ measured here of $+12.8\%$ to $+18.2\%$, the expected range of nitrate $\delta^{18}O$ values produced during nitrification in seawater is -8.3% to -0.7%

(Fig. 6). These $\delta^{18}O_{NO_3}$ values are much lower than those calculated neglecting oxygen isotope effects ($+7.8\%$ to $+12\%$) and are also lower than the $\delta^{18}O$ values of deep ocean nitrate ($+1.5\%$ to $+2.5\%$) that are required to balance the oceanic nitrate $\delta^{18}O$ budget against fractionating nitrogen loss fluxes.

Despite advances made in this study, a large range of values are still possible for the $\delta^{18}O$ of newly produced nitrate (Fig. 6). Whether this represents the true range of possible $\delta^{18}O_{NO_3}$ values or whether field populations have less inherent variability is unknown at this time. The two greatest sources of uncertainty are in the $\delta^{18}O$ of nitrite produced by ammonia oxidation (Casciotti et al. 2010) and the isotope effect for water incorporation by nitrite-oxidizing bacteria (this study).

This study has shown that oxygen isotope effects are expected to play an important role in setting the $\delta^{18}O$ of microbially produced nitrate in the sea, and would be

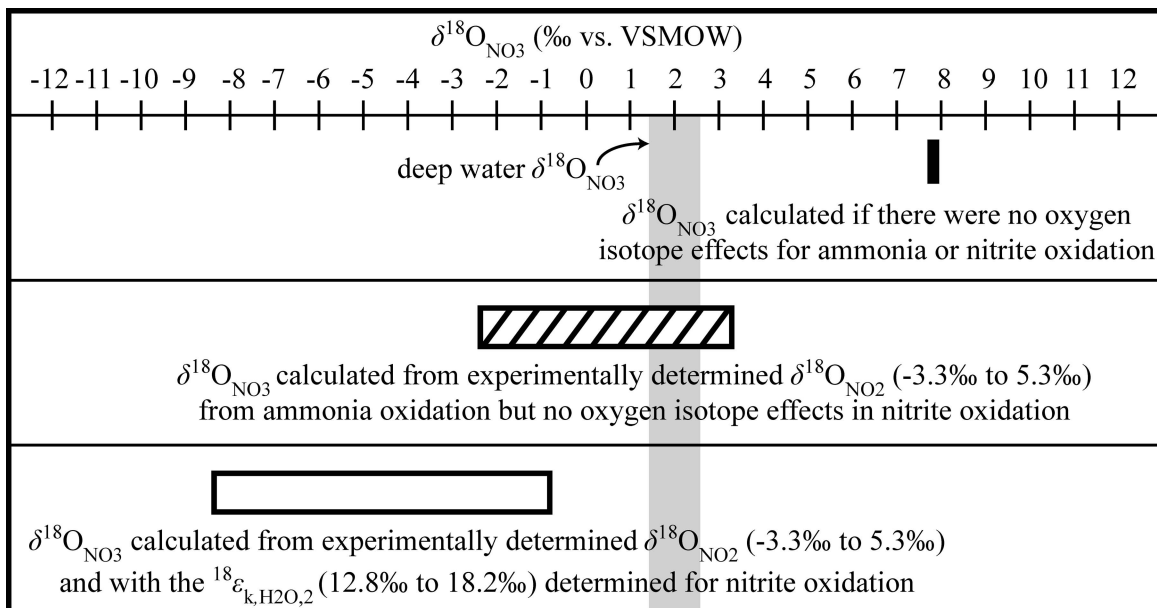


Fig. 6. Recalculation of the $\delta^{18}O$ of nitrate produced from nitrification with a $\delta^{18}O_{O_2}$ value of $+23.5\%$ and a $\delta^{18}O_{H_2O}$ of 0% , and observed $\delta^{18}O_{NO_2}$ values from ammonia-oxidizing bacteria (Casciotti et al. 2010). Incorporating observed oxygen isotope effects for nitrite oxidation causes produced $\delta^{18}O_{NO_3}$ to be lower than that of deep ocean nitrate, which is needed to balance a fixed nitrogen budget using $\delta^{18}O$.

expected as well in other environments. We observed both inter- and intraspecies variability in $^{18}\epsilon_{k,H_2O,2}$. The intraspecies variability can only be attributed to differences in the growth state of the culture at the time of each experiment because all other variables were kept constant between experiments. Although the source(s) of interspecies variability in $^{18}\epsilon_{k,H_2O,2}$ are currently unknown, possible factors that could contribute to this variability include both real enzyme level differences among nitrite-oxidizing systems and apparent differences due to different cytoplasmic $\delta^{18}O_{H_2O}$ values. It is not known with certainty which of these factors might be most important; however, additional experiments with *Nitrospira*, which oxidizes nitrite in the periplasm, rather than in the cytoplasm, could provide insight into the nature of oxygen isotope fractionation during water incorporation.

The ultimate goal of our work is to understand and predict how these isotope effects are expressed by natural assemblages of bacteria and archaea in the ocean. Because there are not yet data on the oxygen isotope systematics of ammonia-oxidizing archaea, which may represent important contributors to the marine nitrogen cycle (Francis et al. 2007), working with these organisms in pure or mixed culture should be a priority. However, because other processes could simultaneously affect the nitrite and nitrate pools in mixed communities, care must be taken in interpreting such data. These concurrent processes might also have isotope effects associated with them, making it difficult to track the role that each process plays in the observed $\delta^{18}O$ variations of nitrate and nitrite pools if multiple parameters are unknown. However, progress is possible on this front with the use of different kinds of manipulative experiments, using ^{18}O -labeled H_2O in much the same way that we have here. These experiments could involve incubating field samples in the dark with or without added ammonia and determining the incorporation of H_2O into the nitrite and nitrate pools. Concurrent analysis of the microbial communities present will help us understand the microbes involved in the observed transformations.

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