

Oxygen isotopic exchange and fractionation during bacterial ammonia oxidation

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Abstract

We examined the oxygen isotopic systematics for ammonia oxidation, the first step in the regeneration of nitrate from ammonium. In particular, oxygen isotopic fractionation and exchange with water were evaluated for their roles in determining the $\delta^{18}\text{O}$ of nitrite produced by four species of ammonia-oxidizing bacteria (AOB). Microbially catalyzed oxygen isotopic exchange between nitrite and water was less than 25% at low cell densities (10^6 cells mL^{-1}) and ammonium concentrations (less than $50 \mu\text{mol L}^{-1}$). The amount of exchange was relatively constant for a given species of ammonia oxidizer but varied between 1% and 25% among the four species tested. The $\delta^{18}\text{O}$ value of nitrite produced at a given water $\delta^{18}\text{O}$ value also varied by nearly 10‰ among the different species. Isotopic fractionation, either during oxygen (O_2) incorporation by ammonia monooxygenase and/or water incorporation by hydroxylamine oxidoreductase plays an important role in setting the $\delta^{18}\text{O}$ of nitrite produced by AOB. This work provides a detailed characterization of the oxygen isotopic systematics of ammonia oxidation by AOB, which will help us better interpret the oxygen isotopic distributions of nitrite, nitrate, and nitrous oxide in terrestrial and aquatic environments.

Nitrogen is an essential macronutrient whose supply can limit primary production and carbon export from the surface ocean on seasonal, annual, decadal, and millennial time scales. Nitrate (NO_3^-) is the primary form of fixed nitrogen in the sea, and much research has focused on quantifying its sources and sinks. Still, present-day mass and isotope budgets of oceanic nitrate contain significant uncertainties, with some estimates yielding a net nitrate loss of approximately 200 Tg N yr^{-1} (Codispoti et al. 2001; Brandes and Devol 2002) and some yielding a nearly balanced budget (Gruber and Sarmiento 1997). These discrepancies reflect uncertainties in the rates of ecologically and biogeochemically important microbial reactions that affect the cycling of both nitrogen and carbon. Resolving the uncertainties in the budget is important for understanding the controls on primary productivity in the past, present, and future. In addition, the turnover of nitrogen controls the production and consumption of nitrous oxide (N_2O), a climatically important trace gas.

Nitrogen isotope ratios in nitrate ($\delta^{15}\text{N}_{\text{NO}_3}$ (‰ vs. AIR) = $\{(^{15}\text{R}_{\text{NO}_3} \div ^{15}\text{R}_{\text{AIR}}) - 1\} \times 1000$, where $^{15}\text{R} = ^{15}\text{N} : ^{14}\text{N}$ and AIR refers to the standard atmospheric N_2) have been used to generate a marine fixed nitrogen budget (Brandes and Devol 2002; Deutsch et al. 2004). Nitrogen fixation introduces new forms of bioavailable nitrogen, while denitrification and anammox in the water column and marine sediments remove bioavailable nitrogen from the deep-ocean inventory. Nitrogen isotope budgets suggest that sedimentary denitrification is much more important than water column denitrification and may account for as much as 80% of total oceanic denitrification, or as much as 280 Tg N yr^{-1} (Brandes and Devol 2002). For the oceanic nitrogen cycle to be balanced, the amount of nitrogen fixation may be higher than current estimates suggest (135 Tg N yr^{-1} ; Deutsch et al. 2007). Verification of this

prediction may be possible by incorporating measurements of the oxygen isotope ratio of oceanic nitrate ($\delta^{18}\text{O}_{\text{NO}_3}$ (‰ vs. VSMOW) = $\{(^{18}\text{R}_{\text{NO}_3} \div ^{18}\text{R}_{\text{VSMOW}}) - 1\} \times 1000$, where $^{18}\text{R} = ^{18}\text{O} : ^{16}\text{O}$ and VSMOW refers to the standard Vienna Standard Mean Ocean Water), bringing oxygen isotopes to bear on the nitrogen cycle.

The application of sensitive new high-throughput methods for simultaneous nitrogen and oxygen isotopic analyses of nitrate (Sigman et al. 2001; Casciotti et al. 2002) has enabled measurements of $\delta^{18}\text{O}_{\text{NO}_3}$ to be made in a variety of oceanographic regions. In deep waters (below $\sim 2500 \text{ m}$), $\delta^{18}\text{O}_{\text{NO}_3}$ values reach consistent values of +1.5‰ to +2.5‰ (Casciotti et al. 2002; Sigman et al. 2009). However, for oxygen isotopic signatures in nitrate to be used effectively as a budgetary constraint in isotope-based models, the isotope effects of processes that produce nitrate (nitrification) and consume it (nitrate reduction during denitrification, assimilation, and dissimilatory nitrate reduction to ammonium) must be understood. Many of the oxygen isotope effects for nitrate consumption have recently been determined (Granger et al. 2004, 2008; Lehmann et al. 2004). However, at this time we lack fundamental understanding about the $\delta^{18}\text{O}$ variations produced by nitrification, which leads to uncertainty in interpretations of the $\delta^{18}\text{O}$ signatures of oceanic nitrate, nitrite, and nitrous oxide.

Nitrification and $\delta^{18}\text{O}_{\text{NO}_3}$ —Nitrification occurs as a two-step process whereby ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) convert ammonia to nitrite and nitrite-oxidizing bacteria (NOB) convert nitrite to nitrate. During the bacterial nitrification process, the biochemical sources of oxygen atoms are dioxygen (O_2) and water (H_2O) (Andersson and Hooper 1983; Kumar et al. 1983). O_2 is incorporated during the oxidation of ammonia to hydroxylamine (NH_2OH), while H_2O is incorporated during the oxidation of both hydroxylamine

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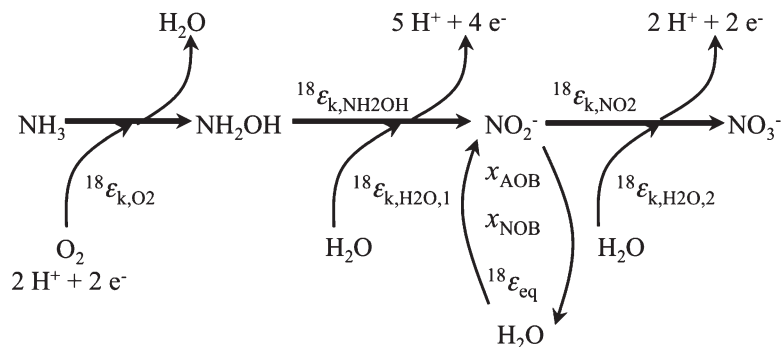


Fig. 1. Oxygen isotopic systematics during the nitrification process. Sources of oxygen atoms (O_2 and H_2O) for ammonia and nitrite oxidation are shown, as well as the predicted isotope effects for NH_2OH and NO_2^- oxidation ($^{18}\epsilon_{k,\text{NH}_2\text{OH}}$ and $^{18}\epsilon_{k,\text{NO}_2^-}$), oxygen atom incorporation ($^{18}\epsilon_{k,\text{O}_2}$, $^{18}\epsilon_{k,\text{H}_2\text{O},1}$, and $^{18}\epsilon_{k,\text{H}_2\text{O},2}$), and exchange ($^{18}\epsilon_{\text{eq}}$). Also shown is the fractional exchange of nitrite oxygen atoms that has been catalyzed by ammonia-oxidizing bacteria (x_{AOB}) and nitrite-oxidizing bacteria (x_{NOB}).

to nitrite and nitrite to nitrate (Fig. 1). While the ratio of 1:2 oxygen atoms from O_2 and H_2O implied by these observations is commonly used to interpret the oxygen isotopic content of nitrate (Kendall 1998; Burns and Kendall 2002; Wankel et al. 2006), the utilization of this ratio involves the assumptions that exchange and fractionation of oxygen isotopes during nitrification are minimal. Although in some cases observed $\delta^{18}\text{O}_{\text{NO}_3^-}$ values do appear to be explained by the biochemical stoichiometry (Mayer et al. 2001), it is important to know if and when this stoichiometry can be used to interpret the $\delta^{18}\text{O}$ values of nitrite, nitrate, and N_2O .

In addition to variations in the $\delta^{18}\text{O}$ of the oxygen atom donors (O_2 and H_2O), factors that might affect the $\delta^{18}\text{O}$ of newly produced nitrate include oxygen isotopic exchange and fractionation. Kinetic isotope effects ($^{18}\epsilon_k$ (‰) = $(^{16}k : ^{18}k - 1) \times 1000$) arise from small differences in the rates at which molecules containing the light isotopes and heavy isotopes react. In the case of oxygen isotopes, ^{16}k is the first-order rate constant for reaction of molecules containing ^{16}O , and ^{18}k is the rate constant for reaction of molecules containing ^{18}O . In Fig. 1, five kinetic isotope effects are shown for (1) selection of hydroxylamine ($^{18}\epsilon_{k,\text{NH}_2\text{OH}}$), (2) selection of nitrite ($^{18}\epsilon_{k,\text{NO}_2^-}$), (3) incorporation of oxygen from O_2 ($^{18}\epsilon_{k,\text{O}_2}$), and (4) and (5) incorporation of oxygen from H_2O during hydroxylamine oxidation and nitrite oxidation ($^{18}\epsilon_{k,\text{H}_2\text{O},1}$ and $^{18}\epsilon_{k,\text{H}_2\text{O},2}$, respectively). In most cases, kinetic isotope fractionation leads to preferential reaction or incorporation of molecules containing the light isotopes ($^{16}k : ^{18}k > 1$ for a “normal” kinetic isotope effect), although for nitrite oxidation this is not necessarily the case (Casciotti 2009; Buchwald and Casciotti in press).

Equilibrium isotope effects, denoted by $^{18}\epsilon_{\text{eq}}$ (‰) = $\{(^{18}\text{R}_1 : ^{18}\text{R}_2) - 1\} \times 1000$ (where $^{18}\text{R}_1$ and $^{18}\text{R}_2$ are the oxygen isotope ratios of two species in equilibrium), arise from differences in the zero point energies of the isotopically substituted molecules and lead to unequal distribution of oxygen isotopes between the species at equilibrium. In Fig. 1, we include the equilibrium isotope effect between NO_2^- and H_2O , which has been determined

for abiotic equilibration of these species at room temperature (Casciotti et al. 2007).

The primary goal of this study was to develop a quantitative understanding of oxygen isotopic signatures in nitrite produced during ammonia oxidation. This information will aid interpretation of $\delta^{18}\text{O}$ variations in nitrate, nitrite, and N_2O in a variety of environmental settings and can be included in oxygen isotope budgets of marine fixed nitrogen, which are analogous and complementary to those generated from nitrogen isotopes.

Methods

Bacterial strains and culture conditions—Batch cultures of AOB *Nitrosomonas* sp. C-113a, *Nitrosococcus oceanii*, *Nitrospira briensis*, and *Nitrosomonas europaea* were maintained in semicontinuous batch culture by periodic (every 2–4 weeks) replacement of 50% of the culture volume with fresh medium. The marine strains C-113a and *N. oceanii* were grown in Watson artificial seawater medium (Watson 1965) containing 5 mmol L^{-1} ammonium chloride (NH_4Cl), while the terrestrial strains *N. briensis* and *N. europaea* were grown in Walker medium (Soriano and Walker 1968), also containing 5 mmol L^{-1} NH_4Cl . For each strain, 1000-mL volumes of maintenance culture were propagated in 2000-mL culture flasks. Cultures were grown in the dark without shaking at 23°C and were neutralized with 1 mL 0.3 mol L^{-1} K_2CO_3 three times prior to harvesting. These strains were chosen to cover known phylogenetic and functional diversity among cultured AOB.

Oxygen isotopic exchange and fractionation experiments—Batch cultures were grown in medium with a range of ^{18}O -labeled H_2O to determine the amount of oxygen isotopic exchange and fractionation for each of the four AOB species listed above. One of the two main variables in these experiments was the partial pressure of oxygen ($p\text{O}_2$), which was tested at two levels: 20.265 kPa (air) and 0.811 kPa. The other primary variable in these experiments was the concentration of initial ammonium ($[\text{NH}_4^+]$),

which was tested at both $5 \mu\text{mol L}^{-1}$ and $50 \mu\text{mol L}^{-1}$. Each experiment was designed to determine oxygen isotopic fractionation and exchange for one combination of AOB species, $[\text{NH}_4^+]$, and pO_2 . All four combinations of the pO_2 and $[\text{NH}_4^+]$ matrix were tested for *Nitrosomonas* sp. C-113a. The other species were tested with $50 \mu\text{mol L}^{-1}$ ammonium and at 20.265 kPa O_2 .

Each experimental culture was grown in a 500-mL glass serum bottle, which contained 100 mL of the appropriate medium (Walker medium for *N. briensis* and *N. europaea* and Watson medium for C-113a and *N. oceanii*) with either $5 \mu\text{mol L}^{-1}$ or $50 \mu\text{mol L}^{-1}$ ammonium. For each pair of pO_2 and $[\text{NH}_4^+]$ conditions, the $\delta^{18}\text{O}$ of the H_2O in the medium ($\delta^{18}\text{O}_{\text{H}_2\text{O}}$) was also adjusted to four different values between -6‰ and $+88\text{‰}$ in replicate bottles. The $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ adjustment was achieved by addition of ^{18}O -enriched H_2O to the base medium before dividing the medium into 100-mL aliquots. One gram of 95 atom % ^{18}O - H_2O (Cambridge Isotope Laboratories) was diluted in 99 mL distilled deionized water to produce a stock solution with $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ of approximately $+5000\text{‰}$. This stock solution was added in 2.5-, 5.0-, or 10.0-mL volumes to 500 mL prepared medium to achieve final media $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ values between -6‰ and $+88\text{‰}$. Each batch of medium was then divided into 100-mL aliquots in four replicate 500-mL bottles, sealed with gray butyl septa, and autoclaved. After autoclaving, each bottle was purged with an $\text{N}_2:\text{O}_2$ mixture for 2 h to achieve the target pO_2 , which was verified through electron-capture gas chromatographic analysis of the headspace gas prior to initiation of the experiment. The bottles were then adjusted to pH 8.2 using 0.1 mol L^{-1} sterile potassium carbonate to minimize abiotic oxygen isotopic exchange between nitrite and H_2O .

Cells for isotopic exchange experiments were harvested from approximately 500 mL of maintenance culture by gentle filtration, then were washed and resuspended in 10 mL of 0.22- μm -filtered fresh or seawater, depending on the culture's salinity requirements. The experiments were initiated by the injection of 1 mL washed cell suspension into replicate 500-mL serum bottles that had been prepared with specified $[\text{NH}_4^+]$, pO_2 , and $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ values (as described above). Uninoculated controls were also incubated at each $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ value with either $5 \mu\text{mol L}^{-1}$ or $50 \mu\text{mol L}^{-1}$ sodium nitrite (NaNO_2) to match the targeted initial $[\text{NH}_4^+]$. In all, a single experimental test of species, $[\text{NH}_4^+]$, and pO_2 contained 16 bottles: four bottles (two replicate inoculated bottles and two replicate uninoculated bottles) at each of the four $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ values.

Time course experiments lasted until the ammonium had been completely oxidized to nitrite, which took approximately 4–10 d. The overall duration of the time course depended on the species and the relative concentrations of O_2 and ammonium in the experiment. Samples (15 mL) were collected at the beginning of the experiment for $[\text{NH}_4^+]$, $[\text{NO}_2^-]$, and $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ analyses. Subsequent time points were subsampled for $[\text{NH}_4^+]$, $[\text{NO}_2^-]$, $\delta^{15}\text{N}_{\text{NO}_2}$, and $\delta^{18}\text{O}_{\text{NO}_2}$ analyses (15 mL total). Liquid volumes removed from the bottles via syringe were replaced with gas injections of the same $\text{N}_2:\text{O}_2$ ratio as the original headspace to maintain a constant pressure and pO_2 in the

bottle. All samples were filtered through a 0.22- μm -pore-size filter immediately after collection. Concentrations and isotopic compositions of nitrite were analyzed within 1 h of sampling to alleviate uncertainty from preservation of $\delta^{18}\text{O}_{\text{NO}_2}$ samples (Casciotti et al. 2007). Samples for $[\text{NH}_4^+]$ and $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ were frozen until analysis.

Chemical and isotopic analyses—Concentrations of ammonium and nitrite were analyzed spectrophotometrically by the indophenol blue assay (Solorzano 1969) and the Greiss–Ilosvay assay (Strickland and Parsons 1972), respectively. Bracketing standards (0 – $50 \mu\text{mol L}^{-1}$) were produced gravimetrically and analyzed in parallel for both ammonium and nitrite.

Isotopic measurements of nitrite ($\delta^{15}\text{N}_{\text{NO}_2}$ and $\delta^{18}\text{O}_{\text{NO}_2}$) were made by conversion of nitrite to N_2O using the “azide method” (McIlvin and Altabet 2005) targeting 10 nmol of nitrite for samples and standards. The N_2O analyte was purged and trapped cryogenically on a custom-built preconcentration system (Casciotti et al. 2002), then released to a Finnigan Delta^{PLUS} XP isotope ratio mass spectrometer. Each sample was analyzed in duplicate and is reported in delta notation relative to AIR ($\delta^{15}\text{N}_{\text{NO}_2}$) or VSMOW ($\delta^{18}\text{O}_{\text{NO}_2}$) by normalizing to nitrite isotopic reference materials N7373, N23, and N10219 (Casciotti et al. 2007), which were analyzed in parallel. Precision for $\delta^{18}\text{O}_{\text{NO}_2}$ measurements is approximately 0.5‰ for replicate analyses (McIlvin and Altabet 2005).

$\delta^{18}\text{O}_{\text{H}_2\text{O}}$ values were measured by equilibration with nitrite and subsequent conversion of nitrite to N_2O using a modified azide method (McIlvin and Casciotti 2006). Each sample was analyzed in duplicate using 0.5-mL water samples, and data are reported in delta notation relative to VSMOW by normalizing to three water standards calibrated in our lab at the Woods Hole Oceanographic Institution (WHOI; McIlvin and Casciotti 2006) (WHOI H_2O -1, $\delta^{18}\text{O}_{\text{H}_2\text{O}} = -5.82 \pm 0.07\text{‰}$ vs. VSMOW; WHOI H_2O -2, $\delta^{18}\text{O}_{\text{H}_2\text{O}} = +18.04 \pm 0.09\text{‰}$ vs. VSMOW; WHOI H_2O -3, $\delta^{18}\text{O}_{\text{H}_2\text{O}} = +41.35 \pm 0.03\text{‰}$ vs. VSMOW). These standards were analyzed in parallel with each set of samples. Precision for $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ measurements is approximately 0.5‰ for replicate analyses (McIlvin and Casciotti 2006).

Data analysis—The $\delta^{18}\text{O}_{\text{NO}_2}$ values produced during ammonia oxidation are expected to depend on the $\delta^{18}\text{O}$ of substrates that are incorporated biochemically (O_2 and H_2O) as well as any isotopic fractionation during oxygen atom incorporation. Subsequent exchange between nitrite and water has the effect of increasing the dependence of $\delta^{18}\text{O}_{\text{NO}_2}$ on $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ and lowering its dependence on $\delta^{18}\text{O}_{\text{O}_2}$. In addition, if there is isotopic fractionation during this equilibration, then the oxygen atom exchange may lead to a systematic offset between $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ and $\delta^{18}\text{O}_{\text{NO}_2}$ (as occurs abiotically; Casciotti et al. 2007). These factors are summarized in Eq. 1, where x_{AOB} is the fraction of nitrite oxygen atoms that have equilibrated with H_2O during ammonia oxidation, $^{18}\epsilon_{\text{k},\text{O}_2}$ is the kinetic isotope effect for O_2 incorporation, $^{18}\epsilon_{\text{k},\text{H}_2\text{O},1}$ is the kinetic isotope effect for H_2O incorporation by hydroxylamine oxidoreductase, and

$^{18}\epsilon_{\text{eq}}$ is the equilibrium isotope effect for nitrite equilibration with H_2O :

$$\delta^{18}\text{O}_{\text{NO}_2} = \left[\frac{1}{2} (\delta^{18}\text{O}_{\text{O}_2} - ^{18}\epsilon_{\text{k},\text{O}_2}) + \frac{1}{2} (\delta^{18}\text{O}_{\text{H}_2\text{O}} - ^{18}\epsilon_{\text{k},\text{H}_2\text{O},1}) \right] (1 - x_{\text{AOB}}) + [\delta^{18}\text{O}_{\text{H}_2\text{O}} + ^{18}\epsilon_{\text{eq}}] (x_{\text{AOB}}) \quad (1)$$

This equation can be rearranged to group terms containing $\delta^{18}\text{O}_{\text{H}_2\text{O}}$, resulting in Eq. 2.

$$\delta^{18}\text{O}_{\text{NO}_2} = \left[\frac{1}{2} (1 + x_{\text{AOB}}) \right] (\delta^{18}\text{O}_{\text{H}_2\text{O}}) + \frac{1}{2} (\delta^{18}\text{O}_{\text{O}_2} - ^{18}\epsilon_{\text{k},\text{O}_2} - ^{18}\epsilon_{\text{k},\text{H}_2\text{O},1}) + (^{18}\epsilon_{\text{eq}}) (x_{\text{AOB}}) \quad (2)$$

Based on Eq. 2, $\delta^{18}\text{O}_{\text{NO}_2}$ is expected to vary linearly with $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ in parallel batch cultures that differ only in their $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ values. The slope of the linear regression yields an expression for x_{AOB} , while the intercept relies on $\delta^{18}\text{O}_{\text{O}_2}$, $^{18}\epsilon_{\text{k},\text{O}_2}$, $^{18}\epsilon_{\text{k},\text{H}_2\text{O},1}$, $^{18}\epsilon_{\text{eq}}$, and x_{AOB} . For low values of x_{AOB} , the intercept is dominated by $\delta^{18}\text{O}_{\text{O}_2}$, $^{18}\epsilon_{\text{k},\text{O}_2}$, and $^{18}\epsilon_{\text{k},\text{H}_2\text{O},1}$. Although we cannot currently separate the isotope effects of O_2 and H_2O incorporation in the intercept term, we can obtain an estimate of the overall kinetic isotopic fractionation for oxygen atom incorporation during ammonia oxidation ($^{18}\epsilon_{\text{k},\text{O}_2} + ^{18}\epsilon_{\text{k},\text{H}_2\text{O},1}$).

Here, we assume that $\delta^{18}\text{O}_{\text{O}_2}$ is similar to atmospheric O_2 , or approximately $+23.5 \pm 1\%$. This is justified from the experimental design, which required that less than 10% of the O_2 was consumed in each bottle. In most cases O_2 consumption was less than 0.3%. Measuring $\delta^{18}\text{O}_{\text{O}_2}$ in our experiments would have allowed more precise determination of $^{18}\epsilon_{\text{k},\text{O}_2} + ^{18}\epsilon_{\text{k},\text{H}_2\text{O},1}$ but would still not have allowed us to separate the two parameters, which together contribute a constant offset to $\delta^{18}\text{O}_{\text{NO}_2}$ (Eq. 2). We also assume that the $^{18}\epsilon_{\text{eq}}$ that has been determined for abiotic oxygen atom exchange between nitrite and water (Casciotti et al. 2007) applies to the enzymatically catalyzed oxygen atom exchange during ammonia oxidation (x_{AOB}) and nitrite oxidation (x_{NOB}). This is justified since enzymes are understood to accelerate the approach to equilibrium but not the equilibrium point of a system. These calculations also assume that oxygen atom exchange occurs between H_2O and NO_2^- but not H_2O and NH_2OH . Exchange of O isotopes between H_2O and NH_2OH probably cannot be ruled out completely. However, studies that determined the oxygen atom source for NH_2OH did not observe exchange between NH_2OH and H_2O in *N. europaea* cultures (Dua et al. 1979; Hollocher et al. 1981). If exchange between H_2O and NH_2OH had occurred in our experiments, it would not change our estimates for the amount of oxygen atom exchange, but it could change the interpretation of the $\delta^{18}\text{O}_{\text{NO}_2}$ vs. $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ intercept.

Results

Time course results for $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ —The results from a typical time course experiment for ammonia oxidation by C-113a are shown in Fig. 2. Ammonium was quantitatively

oxidized to nitrite in each of the experimental bottles, with no significant differences in the rates of ammonia oxidation between bottles with differing $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ values (Fig. 2A). The uninoculated bottles showed no significant change ($p > 0.05$) in the concentrations of ammonium (Fig. 2A) or nitrite (not shown). $\delta^{15}\text{N}_{\text{NO}_2}$ increased by approximately 34‰ over the time course, corresponding roughly to reaction progress (Fig. 2B). These trends are consistent with Rayleigh fractionation for an accumulated product (Mariotti et al. 1981) with expressed isotope effects of $+36.2 \pm 1.4\%$ to $+42.6 \pm 2.2\%$. $\delta^{18}\text{O}_{\text{NO}_2}$ showed comparatively little change over the course of the experiment in a single bottle; however, large differences in $\delta^{18}\text{O}_{\text{NO}_2}$ were observed between bottles, which largely followed the $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ value of the growth medium (Fig. 2C).

The small increase in $\delta^{18}\text{O}_{\text{NO}_2}$ over the time course could have been due to either abiotic exchange or fractional consumption of O_2 in the bottles. Increased $\delta^{18}\text{O}_{\text{O}_2}$ due to fractional consumption of O_2 could lead to increased $\delta^{18}\text{O}_{\text{NO}_2}$ over time. However, in these experiments (conducted with 20.265 kPa O_2 and $50 \mu\text{mol L}^{-1} [\text{NH}_4^+]$), less than 0.3% of the O_2 in the bottle should have been consumed (assuming 1.5 mol of O_2 consumed per mol of ammonia oxidized to nitrite; Bock et al. 1989). Assuming an isotope effect for O_2 consumption of $+18\%$ to $+20\%$ (Bender 1990), the maximum increase in $\delta^{18}\text{O}_{\text{O}_2}$ would be on the order of 0.06‰, leading to a change in the $\delta^{18}\text{O}$ value of produced nitrite of approximately 0.03‰ (given that approximately 50% of the oxygen atoms in nitrite are from O_2), which would be undetectable. Indeed, if the time-dependent increases in $\delta^{18}\text{O}_{\text{NO}_2}$ were due to changes in $\delta^{18}\text{O}_{\text{O}_2}$ rather than exchange with H_2O , then the magnitude of the $\delta^{18}\text{O}_{\text{NO}_2}$ increase would be independent of $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ and would not have been observed in the control bottles. In contrast, we observed a greater increase in $\delta^{18}\text{O}_{\text{NO}_2}$ over time in higher $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ media, with similar magnitudes in both the experimental (Fig. 2C) and the control bottles (Fig. 2D). Together, these observations suggest that the increase in $\delta^{18}\text{O}_{\text{NO}_2}$ over time in a given experimental bottle was due primarily to abiotic nitrite equilibration with H_2O after it had been formed.

Microbially catalyzed oxygen isotopic exchange during ammonia oxidation—Because the change in $\delta^{18}\text{O}_{\text{NO}_2}$ over time in each experimental treatment was minimal and apparently due to abiotic exchange, we used a linear regression of $\delta^{18}\text{O}_{\text{NO}_2}$ vs. time to extrapolate $\delta^{18}\text{O}$ values of the produced nitrite back to the beginning (T_0) of each experiment. These values would represent the $\delta^{18}\text{O}$ value of microbially produced nitrite prior to abiotic exchange. The $\delta^{18}\text{O}_{\text{NO}_2}$ value at T_0 was then plotted against the $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ of the growth medium for each experimental bottle (Fig. 3). Linear regressions of $\delta^{18}\text{O}_{\text{NO}_2}$ vs. $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ for each experiment yield the amount of microbially catalyzed exchange (from the slope) and a “fractionation term” (from the intercept), which includes kinetic and equilibrium isotope effects, as described above (Eq. 2).

The amounts of exchange catalyzed during ammonia oxidation (reported as a percent of nitrite oxygen atoms exchanged) ranged from 1% to 25% for different AOB

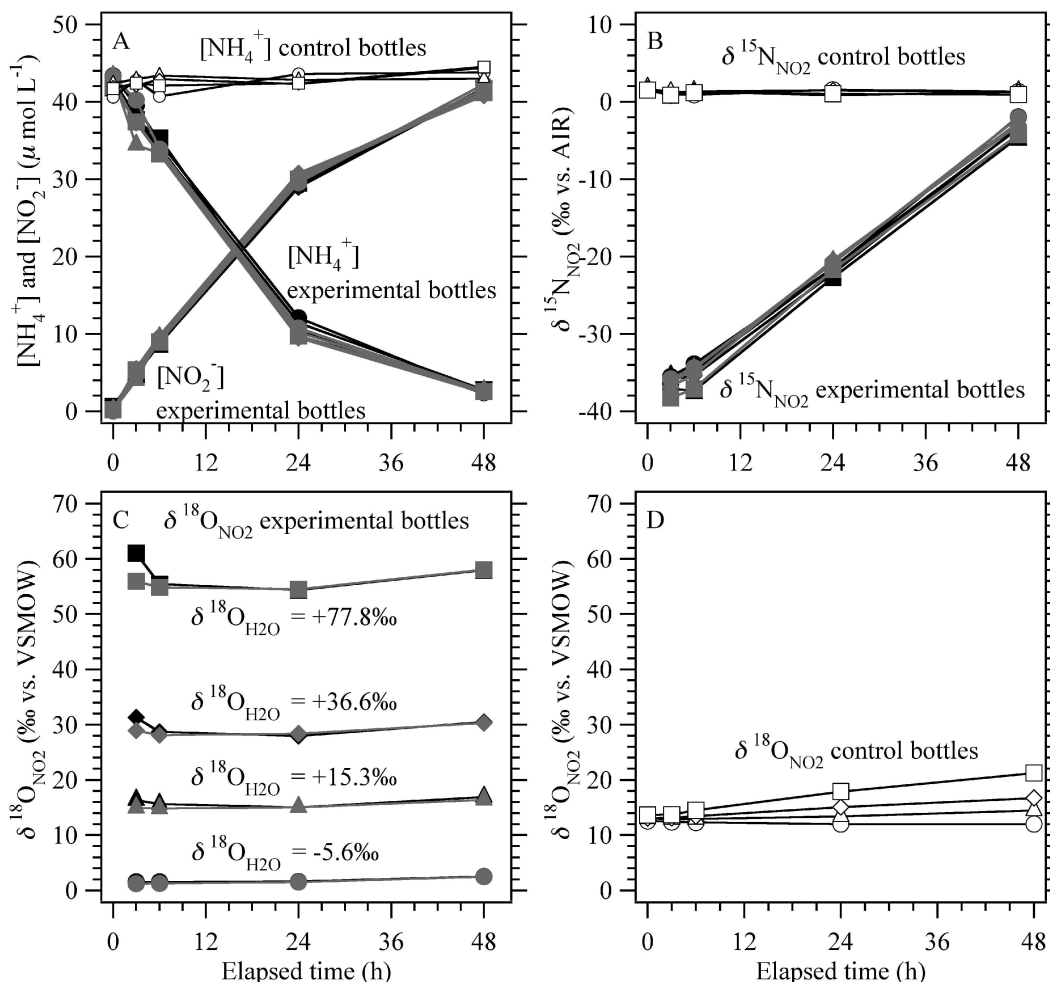


Fig. 2. Time course for ammonia oxidation by *Nitrosomonas* sp. C-113a in a typical experiment. (A) Ammonium and nitrite concentrations show mass balance in experimental (filled symbols) and control (open symbols) bottles. (B) $\delta^{15}\text{N}_{\text{NO}_2}$ (‰ vs. AIR) shows Rayleigh fractionation in the experimental bottles (filled symbols) and no change in control bottles (open symbols). (C) $\delta^{18}\text{O}_{\text{NO}_2}$ (‰ vs. VSMOW) for experimental bottles shows substantial dependence on $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ but little change over the time course at a given $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ value. (D) $\delta^{18}\text{O}_{\text{NO}_2}$ (‰ vs. VSMOW) in control bottles shows a minor amount of abiotic oxygen isotopic exchange between nitrite and H_2O , with $\delta^{18}\text{O}_{\text{NO}_2}$ increases scaling with $\delta^{18}\text{O}_{\text{H}_2\text{O}}$. For each series, the shape of the symbol corresponds to $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ for the medium in which the experiments were conducted. Circles correspond to $\delta^{18}\text{O}_{\text{H}_2\text{O}} = -5.6\%$, triangles correspond to $\delta^{18}\text{O}_{\text{H}_2\text{O}} = +15.3\%$, diamonds correspond to $\delta^{18}\text{O}_{\text{H}_2\text{O}} = +36.6\%$, and squares correspond to $\delta^{18}\text{O}_{\text{H}_2\text{O}} = +77.8\%$. Black and gray filled symbols in (A), (B), and (C) correspond to replicate bottles of the same experimental treatment.

(Table 1). On average, *N. oceanii* catalyzed the greatest amount of exchange, averaging $25 \pm 5\%$, followed by C-113a with $23 \pm 5\%$, *N. europaea* with $11 \pm 3\%$, and finally *N. briensis* with $1 \pm 6\%$. For each species, variations in exchange with ammonia oxidation rate and growth conditions, such as $p\text{O}_2$ (20.265 kPa vs. 0.811 kPa) or initial $[\text{NH}_4^+]$ ($5 \mu\text{mol L}^{-1}$ vs. $50 \mu\text{mol L}^{-1}$), were not significant ($p > 0.05$), and the results are reported as an average of all experiments for a given species.

Oxygen isotopic fractionation during ammonia oxidation—
In addition to differences in catalyzed exchange, the four species of AOB also had different kinetic isotope effects for oxygen atom incorporation. From the intercept of Eq. 2, we can estimate the combined isotopic fractionation for oxygen atom incorporation ($^{18}\epsilon_{\text{k},\text{O}_2} + ^{18}\epsilon_{\text{k},\text{H}_2\text{O},1}$) for the different AOB species if we assume constant values for

$\delta^{18}\text{O}_{\text{O}_2}$ (+23.5‰), $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ (0‰), and $^{18}\epsilon_{\text{eq}}$ (+14‰; Casciotti et al. 2007). The *Nitrosomonas* species had similarly low combined isotope effects (+17.9‰ and +19.3‰), while *N. briensis* and *N. oceanii* had larger combined isotope effects (+30.3‰ and +37.6‰, respectively) (Table 1). At this time, we cannot separate the effects of fractionation during O_2 incorporation ($^{18}\epsilon_{\text{k},\text{O}_2}$) and H_2O incorporation ($^{18}\epsilon_{\text{k},\text{H}_2\text{O},1}$), but together the fractionation factors for these two enzymatic steps led to production of nitrite that ranged in $\delta^{18}\text{O}_{\text{NO}_2}$ from -3.3% to $+5.3\%$ at a $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ value of 0‰ (Table 1). For comparison, we would expect a $\delta^{18}\text{O}_{\text{NO}_2}$ value for produced nitrite of approximately +11.8‰ to +12.3‰ if no isotopic fractionation occurred during O_2 or H_2O incorporation (Eq. 2).

The reason for the observed differences among species is not known at this time. The patterns of oxygen isotope fractionation showed an inverse relationship to bulk

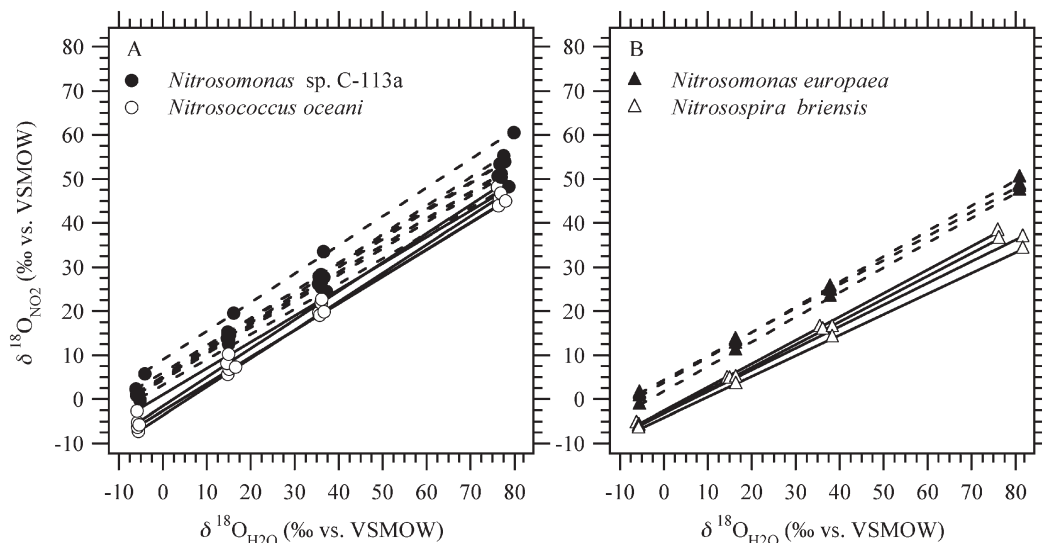


Fig. 3. Dependence of $\delta^{18}\text{O}_{\text{NO}_2}$ on $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ in the growth medium for 23 individual experiments using four species of ammonia-oxidizing bacteria: (A) *Nitrosomonas* C-113a (filled circles) and *Nitrosococcus oceani* (open circles) and (B) *Nitrosomonas europaea* (filled triangles) and *Nitrospira briensis* (open triangles). The slope for each linear regression provides a measure of oxygen isotope exchange between nitrite and H_2O , and the y-intercept yields the isotope effect for oxygen atom incorporation for each experiment.

ammonia oxidation rates, with *Nitrosomonas* species (lower fractionation) oxidizing ammonia more rapidly than *Nitrosococcus* or *Nitrospira* (higher fractionation). However, changes in ammonia oxidation rate caused by growth conditions for a single species did not show a consistent trend. For example, while lowered pO_2 slowed ammonia oxidation rate for all organisms tested, these experiments showed decreased oxygen isotope fractionation in *Nitrosomonas* species but no significant change in fractionation for the other organisms. Further work is needed to separate the effects of oxygen isotope fractionation at O_2 and H_2O incorporation steps in order to better understand the reasons for the species-level differences observed here.

Nitrogen isotopic fractionation during ammonia oxidation—Nitrogen isotope effects for ammonia oxidation calculated from time course $[\text{NO}_2^-]$ and $\delta^{15}\text{N}_{\text{NO}_2}$ data using the Rayleigh accumulated product equation (Mariotti et al. 1981) ranged from +30‰ to +46‰ for individual experiments and showed no statistically significant difference ($p > 0.05$) among species (not shown). There was also no significant correlation between nitrogen isotope effects and oxygen atom exchange, oxidation rate, $[\text{NH}_4^+]$, or pO_2 , although there was a weak negative correlation between nitrogen isotope effects and isotope effects for oxygen atom

incorporation ($r^2 = 0.43$). We conclude that variations in nitrogen isotope effects for ammonia oxidation are driven by factors largely independent of oxygen isotope exchange and fractionation.

Discussion

Constraints on the $\delta^{18}\text{O}_{\text{NO}_3}$ source signature—While oxygen isotopic measurements of nitrate in seawater have been possible only in recent years, their interpretation has already provided important insights into the cycling of nitrate in the marine environment, such as diagnosing the input of newly fixed nitrogen in oxygen deficient waters (Sigman et al. 2005), detecting the influence of euphotic zone nitrification (Wankel et al. 2007), and examining the reoxidation of nitrite to nitrate on the fringes of oxygen-deficient zones (Sigman et al. 2005; Casciotti and McIlvin 2007). These studies have made the assumption that nitrification produces nitrate with a $\delta^{18}\text{O}$ value near that of ambient H_2O (0‰ to +3‰), but the lack of specific information about the oxygen isotopic systematics of nitrification has contributed uncertainty to many of these interpretations.

The oxygen isotopic systematics for nitrification also provide information needed for using $\delta^{18}\text{O}_{\text{NO}_3}$ to constrain the oceanic nitrogen budget. Nitrification is the process

Table 1. Oxygen isotopic fractionation and exchange during ammonia oxidation.

Species	$\delta^{18}\text{O}_{\text{NO}_2}$ vs. $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ slope (unitless)	$\delta^{18}\text{O}_{\text{NO}_2}$ vs. $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ y-intercept (‰)	x_{AOB} (exchange fraction)	$^{18}\epsilon_{\text{k},\text{O}_2} + ^{18}\epsilon_{\text{k},\text{H}_2\text{O},1}$ (‰)
<i>Nitrosomonas</i> sp. C-113a	0.61 ± 0.03	$+5.3 \pm 1.7$	0.23 ± 0.05	$+17.9 \pm 3.6$
<i>Nitrosomonas europaea</i>	0.56 ± 0.01	$+3.5 \pm 1.1$	0.11 ± 0.03	$+19.3 \pm 2.9$
<i>Nitrosococcus oceani</i>	0.62 ± 0.02	-1.9 ± 1.8	0.25 ± 0.05	$+37.6 \pm 6.1$
<i>Nitrospira briensis</i>	0.50 ± 0.03	-3.3 ± 0.6	0.01 ± 0.06	$+30.3 \pm 1.0$

that constitutes the source signature for $\delta^{18}\text{O}_{\text{NO}_3}$, while nitrate reduction during denitrification and nitrate assimilation constitute sinks for oxygen atoms in nitrate. Given that consumption processes are expected to raise the $\delta^{18}\text{O}$ of deep-ocean nitrate relative to the input from nitrification, it can be argued that the $\delta^{18}\text{O}$ value of newly produced nitrate must be lower than the average deep-ocean $\delta^{18}\text{O}_{\text{NO}_3}$ value of approximately +2.5‰ (Sigman et al. 2009).

While the sources of oxygen atoms to nitrate have been shown to be from O_2 and H_2O (in a 1:2 ratio; Andersson and Hooper 1983; Kumar et al. 1983), direct incorporation of these substrates with no isotopic fractionation or exchange would lead to production of nitrate with a $\delta^{18}\text{O}$ value of +7.8‰ to +12‰, depending on $\delta^{18}\text{O}_{\text{O}_2}$ values, which in seawater can range from +23.5‰ to +40‰ vs. VSMOW (Bender 1990; Levine et al. 2009). Oxygen isotopic exchange between nitrite and H_2O is a mechanism that has been proposed for lowering the $\delta^{18}\text{O}$ value of newly produced nitrite relative to the oxygen atom substrates and therefore subsequently lowering the expected $\delta^{18}\text{O}$ of newly produced nitrate to the observed values (Casciotti et al. 2002; Sigman et al. 2005). However, recent work has shown that a large equilibrium isotope effect is associated with oxygen atom exchange between nitrite and H_2O (Casciotti et al. 2007), which implies that exchange would drive $\delta^{18}\text{O}_{\text{NO}_2}$ toward +14‰ rather than 0‰. As shown in Eq. 3, only a large kinetic isotope effect for H_2O incorporation during nitrite oxidation ($^{18}\epsilon_{\text{k,H}_2\text{O},2} > +20\%$) could then allow for production of nitrate with a $\delta^{18}\text{O}$ value less than +2.5‰ after full equilibration:

$$\delta^{18}\text{O}_{\text{NO}_3} = \frac{2}{3}(\delta^{18}\text{O}_{\text{NO}_2}) + \frac{1}{3}(\delta^{18}\text{O}_{\text{H}_2\text{O}} - ^{18}\epsilon_{\text{k,H}_2\text{O},2}) \quad (3)$$

Here we show that the nitrite produced by AOB undergoes less than 25% enzymatically catalyzed exchange and that the nitrite produced (in water with $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ of 0‰) has $\delta^{18}\text{O}_{\text{NO}_2}$ values that are lower (ranging from -3.3‰ to +5.3‰) than predicted from biochemical stoichiometry. Notably, these $\delta^{18}\text{O}_{\text{NO}_2}$ values were achieved largely through isotopic fractionation during oxygen atom incorporation rather than through oxygen atom exchange.

Oxygen isotopic variations in deep-ocean nitrate—Our data, which suggest that less than 25% exchange occurs during ammonia oxidation, may be surprising given the lack of significant $\delta^{18}\text{O}_{\text{NO}_3}$ variations in deep water. Despite large variations in $\delta^{18}\text{O}_{\text{O}_2}$, the small variations in deep-ocean $\delta^{18}\text{O}_{\text{NO}_3}$ have been used to argue that little of the isotopic input from O_2 is retained in the nitrate pool and that near complete oxygen atom exchange occurs during nitrification in the sea (Casciotti et al. 2002; Sigman et al. 2009). Using a multibox model of the ocean, Sigman and colleagues (2009) examined the consequences of $\delta^{18}\text{O}_{\text{O}_2}$ variation on deep-ocean $\delta^{18}\text{O}_{\text{NO}_3}$ gradients at two levels of oxygen atom exchange: full exchange (resulting in no remaining O_2 signal) and 50% exchange (resulting in O_2 providing one in six of the oxygen atoms in nitrate after nitrite oxidation).

The results of Sigman and colleagues (2009) suggested that retention of the oxygen atom input from O_2 should be less than one in six of the oxygen atoms in nitrate, equivalent to an exchange of greater than 50%. However, our results show that for AOB operating at moderate $[\text{NH}_4^+]$ (5–50 $\mu\text{mol L}^{-1}$) and cell densities (10⁶ cells mL⁻¹), approximately one in four of the oxygen atoms remaining in nitrate originates from O_2 , a case that retains more O_2 than either scenario considered by Sigman et al. (2009). The relatively low amount of exchange observed here does remove some dependence of $\delta^{18}\text{O}_{\text{NO}_2}$ and $\delta^{18}\text{O}_{\text{NO}_3}$ on variations in $\delta^{18}\text{O}_{\text{O}_2}$ but does not eliminate it altogether. We do not currently have an explanation for the apparent discrepancy between our observations and the modeling constraints from Sigman et al. (2009). Perhaps the existing full-depth profiles of $\delta^{18}\text{O}_{\text{NO}_3}$ are too spatially limited to resolve the expected $\delta^{18}\text{O}_{\text{NO}_3}$ gradients. Additional deep-ocean $\delta^{18}\text{O}_{\text{NO}_3}$ measurements, such as those provided by the international GEOTRACES program, may provide more information with which to interpret deep-ocean gradients in $\delta^{18}\text{O}_{\text{NO}_3}$ relative to $\delta^{18}\text{O}_{\text{O}_2}$.

Our results showing less than 25% oxygen atom exchange during ammonia oxidation may also seem surprising given previous work reporting much higher levels of exchange (Andersson et al. 1982). However, high cell densities and high $[\text{NH}_4^+]$ in these earlier experiments could have led to local decreases in pH, which would accelerate abiotic oxygen atom exchange. Indeed, it was later shown that the amount of exchange could be reduced using lower cell densities (Andersson and Hooper 1983). In our experiments, even lower $[\text{NH}_4^+]$ (5–50 $\mu\text{mol L}^{-1}$) and cell densities ($\sim 10^6$ mL⁻¹) were used. In addition, pH was carefully controlled, and the effects of abiotic exchange were carefully evaluated using parallel control bottles. We argue that although the AOB cell densities and $[\text{NH}_4^+]$ are still higher than most marine settings, the amount of exchange observed here is likely to be more representative of that catalyzed by AOB than the high levels reported in earlier studies.

We also recognize that AOA, which are ubiquitous and abundant in ocean waters (Francis et al. 2007), may have different oxygen isotopic systematics than AOB and will need to be studied in order to gain a complete understanding of oxygen isotopic variations in nitrite, nitrate, and N_2O . Given that H_2O incorporation (and presumably exchange) is catalyzed by the enzyme hydroxylamine oxidoreductase (HAO) in AOB (Andersson et al. 1982; Andersson and Hooper 1983), it should be noted that the AOA *Nitrosopumilis maritimus* apparently lacks an HAO analogue (Klotz et al. 2008) and may have an alternate mechanism for production of nitrite from ammonium. This potential biochemical difference could yield different oxygen isotopic systematics (fractionation and exchange) for nitrite production by AOA, compared with AOB. However, if O_2 remains a substrate in the reaction, oxygen atom exchange with water would again be required to explain the small gradients in deep-ocean $\delta^{18}\text{O}_{\text{NO}_3}$. In order to determine with certainty the appropriate $\delta^{18}\text{O}_{\text{NO}_3}$ signature from nitrification to include in interpretations of $\delta^{18}\text{O}_{\text{NO}_3}$, additional laboratory and field experi-

ments with ammonia- and nitrite-oxidizing microbes are needed.

Nitrate source apportionment based on $\delta^{18}\text{O}_{\text{NO}_3}$ —In terrestrial and estuarine ecosystems, $\delta^{18}\text{O}_{\text{NO}_3}$ measurements have been used to distinguish between atmospheric and microbially derived nitrate sources to surface water and groundwater (Kendall 1998; Burns and Kendall 2002; Barnes et al. 2008). Key parameters in these analyses include the $\delta^{18}\text{O}_{\text{NO}_3}$ signatures of each source. Atmospheric deposition typically has high $\delta^{18}\text{O}_{\text{NO}_3}$ values, ranging from +20‰ to +90‰ (Kendall 1998; Hastings et al. 2003), while microbial nitrate production via nitrification is generally assumed to have a lower $\delta^{18}\text{O}_{\text{NO}_3}$ value that reflects the sources of O atoms in NO_3^- (O_2 and H_2O) in a 1:2 ratio. In cases where observed nitrate production has not fit with the expected 1:2 ratio from $\delta^{18}\text{O}_{\text{O}_2}$ and $\delta^{18}\text{O}_{\text{H}_2\text{O}}$, interpretation has focused on the influence of microscale variability in $\delta^{18}\text{O}$ values of O_2 and H_2O (Burns and Kendall 2002; Kendall and Aravena 2002).

While the possibility of O atom exchange between NO_2^- and water has been proposed as a mechanism for $\delta^{18}\text{O}_{\text{NO}_3}$ variations, the amount of exchange occurring in a given system has not routinely been determined. In addition, while isotopic fractionation during nitrification has been discussed as a potential factor in setting $\delta^{18}\text{O}$ values of nitrite and nitrate in some early work (Mayer et al. 2001; Kendall and Aravena 2002), the isotopic fractionation has generally been assumed to be negligible, and the magnitude of this effect has not been determined experimentally. Based on the results of the current study, it is important to consider the roles of both oxygen isotopic exchange and fractionation, in addition to variations in the $\delta^{18}\text{O}$ values of O_2 and H_2O substrates, in interpreting the fluxes of microbially produced nitrate. Isotopic fractionation during oxygen atom incorporation may explain why the observed $\delta^{18}\text{O}_{\text{NO}_3}$ values in the Connecticut River watershed (Barnes et al. 2008) and San Joaquin delta (Wankel et al. 2006) were occasionally lower than expected from concurrent $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ and $\delta^{18}\text{O}_{\text{O}_2}$ values. With a lower $\delta^{18}\text{O}_{\text{NO}_3}$ value for microbially produced NO_3^- , less nitrification would also be needed to explain a low $\delta^{18}\text{O}_{\text{NO}_3}$ value obtained by mixing between atmospheric and microbial sources.

Nitrous oxide production by AOB—In addition to tracing the role of nitrification in nitrite and nitrate cycling, the results for oxygen isotopic exchange and fractionation during ammonia oxidation are important for understanding the production of N_2O , a climatically important trace gas. N_2O is produced largely by microbial processes of nitrification and denitrification and is also consumed by denitrification (Stein and Yung 2003). The roles of nitrification and denitrification as sources of N_2O have also been inferred on the basis of nitrogen and oxygen isotopic measurements ($\delta^{15}\text{N}_{\text{N}_2\text{O}}$ and $\delta^{18}\text{O}_{\text{N}_2\text{O}}$), although the use of $\delta^{18}\text{O}_{\text{N}_2\text{O}}$ to distinguish sources has been hampered by a lack of isotope effect estimates for N_2O production by nitrification and denitrification processes (Perez et al. 2000).

Experiments using ^{18}O -labeled H_2O (Wrage et al. 2005; Kool et al. 2007) have been more successful than natural abundance $\delta^{18}\text{O}_{\text{N}_2\text{O}}$ measurements at distinguishing terrestrial sources of N_2O because high levels of ^{18}O enrichment allow these studies to focus on oxygen atom sources rather than isotopic fractionation. However, knowledge about the exchange of oxygen atoms with water during the nitrification process would be helpful to these studies as well. Concurrent measurement of $\delta^{18}\text{O}_{\text{NO}_2}$ and $\delta^{18}\text{O}_{\text{NO}_3}$ would help to constrain the extent of exchange. Moreover, with additional information about the oxygen isotopic fractionation during ammonia oxidation, distinctions between sources of N_2O may be possible based on natural abundance $\delta^{18}\text{O}_{\text{N}_2\text{O}}$ measurements.

Source apportionment of N_2O in the ocean has made use of $\delta^{18}\text{O}_{\text{N}_2\text{O}}$ variations relative to the potential oxygen atom sources (O_2 , H_2O , NO_3^-). N_2O in seawater has routinely been observed to possess $\delta^{18}\text{O}$ values that are higher than the sources of oxygen, although the reasons for this isotopic enrichment are not well understood. Two studies (Ostrom et al. 2000; Popp et al. 2002) used the $\delta^{18}\text{O}$ difference between N_2O and O_2 ($\Delta^{18}\text{O}$ (‰) = $\delta^{18}\text{O}_{\text{N}_2\text{O}} - \delta^{18}\text{O}_{\text{O}_2}$) to infer sources of N_2O at Station ALOHA in the North Pacific Subtropical Gyre. $\delta^{18}\text{O}_{\text{N}_2\text{O}}$ values were approximately 22‰ higher than $\delta^{18}\text{O}_{\text{O}_2}$ at most depths in the water column, although $\Delta^{18}\text{O}$ decreased to values as low as +12‰ between 350 m and 500 m. They argued that this decrease was due to a greater influence of H_2O on $\delta^{18}\text{O}_{\text{N}_2\text{O}}$, representing a greater role for nitrifier denitrification in N_2O production in this depth range. This interpretation is consistent with the oxygen isotope systematics for ammonia oxidation determined in this study. However, information on the oxygen isotopic systematics of N_2O production during nitrification is needed to fully interpret the magnitude of the $\delta^{18}\text{O}$ difference between N_2O and O_2 . The experimental observations reported here provide relevant information for N_2O source determinations, but studies focused on fractionation of oxygen isotopes during N_2O production and consumption by individual microbial pathways are still needed.

Acknowledgments

The authors gratefully acknowledge funding support from the National Science Foundation Division of Ocean Sciences, grant 05-26277, and the comments of two anonymous reviewers.

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Associate editor: Robert D. Bidigare

Received: 12 July 2009

Accepted: 25 September 2009

Amended: 08 December 2009