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Oxygen Isotopes in Nitrite: Analysis, Calibration, and Equilibration

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Nitrite is a central intermediate in the nitrogen cycle and can persist in significant concentrations in ocean waters, sediment pore waters, and terrestrial groundwaters. To fully interpret the effect of microbial processes on nitrate (NO_3^-), nitrite (NO_2^-), and nitrous oxide (N_2O) cycling in these systems, the nitrite pool must be accessible to isotopic analysis. Furthermore, because nitrite interferes with most methods of nitrate isotopic analysis, accurate isotopic analysis of nitrite is essential for correct measurement of nitrate isotopes in a sample that contains nitrite. In this study, nitrite salts with varying oxygen isotopic compositions were prepared and calibrated and then used to test the denitrifier method for nitrite oxygen isotopic analysis. The oxygen isotopic fractionation during nitrite reduction to N_2O by *Pseudomonas aureofaciens* was lower than for nitrate conversion to N_2O, while oxygen isotopic exchange between nitrite and water during the reaction was similar. These results enable the extension of the denitrifier method to oxygen isotopic analysis of nitrite (in the absence of nitrate) and correction of nitrate isotopes for the presence of nitrite in "mixed" samples. We tested storage conditions for seawater and freshwater samples that contain nitrite and provide recommendations for accurate oxygen isotopic analysis of nitrite by any method. Finally, we report preliminary results on the equilibrium isotope effect between nitrite and water, which can play an important role in determining the oxygen isotopic value of nitrite where equilibration with water is significant.

Nitrite (NO_2^-) is an intermediate species in the redox cycling of nitrogen and is produced and consumed by microbial processes such as nitrification, denitrification, nitrate assimilation, dissimilatory nitrate reduction to ammonium, and anaerobic ammonium oxidation. As an intermediate in these transformations, nitrite may accumulate in regions of the ocean where processes responsible for its production and consumption are decoupled, such as in the primary nitrite maximum at the base of the euphotic zone[1−4] or the secondary nitrite maximum within oceanic oxygen-deficient zones with active denitrification.[5−13] Similarly, nitrite can accumulate to varying degrees in soils with varying redox states or groundwater and surface water systems where rates of nitrite production and consumption are not in steady state.[14,15] Active cycling of nitrite in these systems is likely to impart a wide range of isotopic signatures in nitrite, as well as downstream products such as nitrate and N_2O, that reflect the balance of underlying microbial processes. Incorporating isotopic measurements of nitrite into biogeochemical models may thus ultimately help constrain nitrogen cycling in systems where it is a significant fraction of the total nitrogen oxide pool.

Analyses of $^{15}N/^{14}N$ ratios in nitrite have been made for decades and have provided important information about rates of nitrogen cycling in aquatic environments.[1,2,10,15−26] Recent meth-

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odological advancements have also enabled $\delta^{15}N$ analysis of nanomole levels of nitrite at natural abundance levels,[27,28] but the analysis of the oxygen isotopic content of nitrite has lagged nitrogen isotopic analysis because of problems associated with sample analysis, standardization, and storage. The focus of this study is on solving these problems to achieve accurate analyses of oxygen isotopic ratios in nitrite. Critical to the accurate analysis of $\delta^{18}O$ in nitrite by the denitrifier method[27,29] is an understanding of the behavior of nitrite during reduction to N_2O. During the reduction of nitrate and nitrite to N_2O, there are two fates for oxygen atoms: transfer to the subsequent nitrogen oxide pool or loss as water.[13,28] This "branching" of pathways for oxygen atoms occurs with isotopic discrimination, such that light isotopes of oxygen are preferentially lost, leaving the analyte pools progressively enriched in ^{18}O along the reaction sequence. This was observed and described previously for nitrate reduction to N_2O in the denitrifier method.[29] However, as nitrite reduction to N_2O represents a smaller fractional loss of oxygen atoms, the branching isotope effect for nitrite reduction to N_2O is expected to be lower. Differential branching fractionation between nitrate and N_2O and nitrite and N_2O can cause underestimation of nitrate $\delta^{18}O$ values in samples that contain nitrite, if the nitrite contribution to $\delta^{18}O$ is not properly accounted for. This consideration applies not only to the denitrifer method but also to the azide method,[28] which relies on cadmium reduction of nitrate to nitrite prior to nitrite reaction with sodium azide to form N_2O.

Independent of analytical issues, another complication for the interpretation of measured oxygen isotopic ratios in nitrite (by any method) is the ready exchange of oxygen atoms between nitrite and water. While oxygen atoms in nitrate are relatively stable with respect to exchange with water, the oxygen atoms in nitrite are more labile, as reported at high concentrations of nitrite and low pH.[28,30−32] Information about the rate of exchange of oxygen atoms in nitrite (or the protonated form, nitrous acid) with water under more environmentally relevant nitrite concentrations and pH values, however, is more limited.

In order to address analysis, storage, and standardization issues by tracking the isotopic behavior of nitrite, we describe the production and calibration of nitrite salts with a range in $\delta^{18}O$ values of +4.5 to +88.5‰ versus VSMOW and $\delta^{15}N$ from −79.6 to +3.7‰ versus air. These salts were used to calibrate nitrite $\delta^{15}N$ and $\delta^{18}O$ measurements by conversion to N_2O, using the denitrifier method. We also report the results of experiments designed to test the stability of oxygen isotopic ratios in nitrite stored under various pH and temperature conditions in freshwater and seawater. This is important because, in addition to affecting actual distributions of oxygen isotopes in nitrogen oxides, exchange has the potential to affect apparent (measured) distributions of oxygen isotopes in nitrite if samples are stored improperly. Given the importance of oxygen isotopic exchange between nitrite and water, we also investigated the equilibrium isotope effect for this reaction. The equilibrium data have important implications not only for measurement and calibration of oxygen isotopic data in nitrite and nitrate but also for understanding and interpreting oxygen isotopic signatures of nitrification and denitrification intermediates in regions where these processes naturally occur.

EXPERIMENTAL SECTION

Terms and Definitions. Unless otherwise stated, oxygen isotopic ratios are expressed here in delta notation, normalized to VSMOW: $\delta^{18}O = [^{18}R_{sample}/^{18}R_{VSMOW} - 1]$, where $^{18}R = n(^{18}O)/n(^{16}O)$, and are generally reported in units of per mil (‰) by multiplying by a factor of 1000. Branching isotope effects (i.e., $^{18}\epsilon_{b,NO_2} = {}^{18}R_{N_2O}/{}^{18}R_{NO_2} - 1$) and equilibrium isotope effects (i.e., $^{18}\epsilon_{eq,NO_2} = {}^{18}R_{NO_2}/{}^{18}R_{H_2O} - 1$) are also reported in units of per mil by multiplying by a factor of 1000.

Preparation and Calibration of Nitrite Salts with Varying Isotopic Composition. Four nitrite salts with different isotopic compositions were prepared and analyzed as part of this study: RSIL-N20 (KNO_2), RSIL-N23 ($NaNO_2$), RSIL-N7373 ($NaNO_2$), and RSIL-N10219 ($NaNO_2$). Both N20 and N23 are off the shelf reagents. N7373 was prepared by dissolving 100 g of N20 in 25 mL of distilled, deionized (DI) water and then passing the solution through a column containing AG50W-X8 cation exchange resin in the Na form. To prepare N10219 (a nitrite salt with a high $\delta^{18}O$ value), normal reagent $NaNO_2$ was equilibrated with ^{18}O-enriched water. The water was prepared gravimetrically by mixing normal meteoric water ($\delta^{18}O = -6.5‰$) with water containing 0.803 at. % ^{18}O (Icon Stable Isotopes), giving a $\delta^{18}O$ of ∼ +90‰ versus VSMOW. After $NaNO_2$ was added to the water (4.3 mol L^{-1}), the resulting solution had a pH of 4.0. After 12 days of equilibration at room temperature, 3 mL of the solution was removed and dried overnight at 60 °C to recover the nitrite salt for isotopic analysis to test the progress of the equilibration. After an additional three weeks, the remainder of the solution was evaporated to dryness by heating at 60 °C overnight. The dried salt (designated RSIL-N10219) was ground, homogenized, and stored in Teflon-sealed glass vials under desiccation.

Oxygen isotopic analyses of these four nitrite salts were carried out by online high-temperature reaction with granular carbon to produce CO, which was analyzed by continuous-flow isotope ratio mass spectrometry (designated CO-CFIRMS), as described in Böhlke et al.[33] Samples containing ∼4 μmol of $NaNO_2$ were weighed into 3.5 × 5 mm silver foil capsules (Costech), which were loaded into a Costech "zero-blank" autosampler upstream of a Thermo-Finnigan thermochemical elemental analyzer operated at 1325 °C. Gases produced in the reactor were passed through a mol-sieve gas chromatograph to separate N_2 from CO. The N_2 peak was diverted to waste and replaced with pure He to minimize formation of NO in the ion source, and the CO peak was monitored at m/z 28 and 30. To reference these analyses on the VSMOW scale, nitrate isotopic reference materials were analyzed along with the nitrite samples and all data were normalized to yield $\delta^{18}O_{VSMOW}$ values of −27.9‰ for USGS34, +25.6‰ for IAEA-N3, and +57.5‰ for USGS35.[33] The $\delta^{15}N$ values of nitrite salts were calibrated independently by offline reaction

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with low-blank Cu + Cu_2O and CaO in sealed tubes to produce N_2,[34] followed by dual-inlet isotope ratio mass spectrometry on the N_2, normalized to values of +4.7‰ for IAEA-N3 and +180.0‰ for USGS32.[35]

Nitrite Isotopic Analysis by the Denitrifier Method. Nitrite samples were analyzed by the denitrifier method using *Pseudomonas aureofaciens*, a strain of denitrifier that reduces nitrate and nitrite quantitatively to N_2O.[29] Bacterial cultures and sample vials were prepared as described for the denitrifier method,[27,29] and samples were added to yield a consistent amount of N_2O analyte, matched with reference materials of the same amount (typically 10 nmol of N_2O generated from 20 nmol of NO_2^-). This matching of sample and reference amounts allows the application of a blank correction and normalization factors developed from the reference materials analyzed in parallel. The N_2O analyte was purged and trapped from each vial on an automated sample preparation and purification system described previously,[29] as modified by Coplen and others[36] (and T.B. Coplen, USGS, personal communication).

Each individual sample or standard analysis is referenced to a pulse of N_2O reference gas introduced to the open split[37] prior to elution of the sample peak. These provisional $\delta^{18}O$ values of nitrite-derived N_2O are then converted provisionally to the VSMOW reference scale given the independently measured $\delta^{18}O$ value of the N_2O reference tank using the following equation: $\delta^{18}O_{m,NO_2} = \delta^{18}O_{sample/tank} + \delta^{18}O_{tank/VSMOW} + (\delta^{18}O_{sample/tank} \times \delta^{18}O_{sample/VSMOW})$. This yields the measured $\delta^{18}O$ of the nitrite-derived N_2O on the VSMOW scale, which can be expressed in per mil notation by multiplying by 1000. To calculate the $\delta^{18}O$ value of the original nitrite material, an additional calibration step is required, taking into account oxygen isotopic fractionation, isotopic exchange, and blank effects. By analogy with the equations described previously for nitrate $\delta^{18}O$ analysis by the denitrifier method,[29] a similar equation was developed to describe the measured $\delta^{18}O$ of nitrite:

$$\delta^{18}O_{m,NO_2}m = (\delta^{18}O_{s,NO_2} + {}^{18}\epsilon_{B,NO_2})s(1 - x_{NO_2}) + (\delta^{18}O_{H_2O} + {}^{18}\epsilon_{eq},NO_2 + {}^{18}\epsilon_{B,NO_2})sx_{NO_2} + \delta^{18}O_b b \quad (1)$$

Equation 1 describes the dependence of $\delta^{18}O_{m,NO_2}$, the measured $\delta^{18}O$ of nitrite-derived N_2O (vs VSMOW), on $\delta^{18}O_{s,NO_2}$, the original $\delta^{18}O$ of the nitrite material (vs VSMOW), as well as m, the amount of N_2O analyte (in nmol of O), which includes s, the amount of N_2O that originates from nitrite (in nmol of O), and b, the amount of N_2O blank (in nmol of O). The branching isotope effect (${}^{18}\epsilon_{B,NO_2}$) represents the isotopic fractionation that occurs as a result of preferential loss of ^{16}O during nitrite reduction to N_2O. $\delta^{18}O_{H_2O}$ and $\delta^{18}O_b$ are the $\delta^{18}O$ values (vs VSMOW) of the ambient water (bacterial growth medium plus aqueous sample) and the blank, respectively, and x_{NO_2} is the fraction of oxygen atoms in nitrite that exchange with water during the analysis. Equation 1 is distinct from that developed previously for nitrate[29] in the modification of the exchange term by the equilibrium isotope effect between nitrite and water (${}^{18}\epsilon_{eq,NO_2}$, see Terms and Definitions). The isotopic effects of blank, exchange, and branching fractionation on measured $\delta^{18}O$ of nitrate and nitrite are shown schematically in Figure 1. An analogous equation for nitrate analysis was modified from the original description[29] to include ${}^{18}\epsilon_{eq,NO_2}$, which affects measured nitrate $\delta^{18}O$ values in the same way in which it affects measured nitrite $\delta^{18}O$ values:

$$\delta^{18}O_{m,NO_3}m = (\delta^{18}O_{s,NO_3} + {}^{18}\epsilon_{B,NO_3} + {}^{18}\epsilon_{B,NO_2})s(1-x_{NO_3}) + (\delta^{18}O_{H_2O} + {}^{18}\epsilon_{eq,NO_2} + {}^{18}\epsilon_{B,NO_2})sx_{NO_3} + \delta^{18}O_b b \quad (2)$$

We considered using ${}^{18}\epsilon_{eq,NO_3}$, the isotope effect for nitrate−water equilibration[33] in eq 2, but ultimately assumed that, during nitrate reduction to N_2O, most of the isotopic exchange in the reaction occurs between water and nitrite, not nitrate. This assumption was verified by comparing the amounts of oxygen isotopic exchange during nitrate and nitrite analysis (see below). The original equation[29] was also altered to divide the branching isotope effect, ϵ, into individual isotope effects ${}^{18}\epsilon_{B,NO_3}$ between nitrate and nitrite, and ${}^{18}\epsilon_{B,NO_2}$ between nitrite and N_2O (Figure 1). Given the use of delta notation, these equations are approximations, but they yield excellent results for the work described here.

The denitrifier method relies on consistent isotopic fractionation, exchange, and blank within a batch of samples for accurate $\delta^{18}O$ analyses. Importantly, the values of these parameters for nitrite conversion to N_2O are not necessarily the same as for

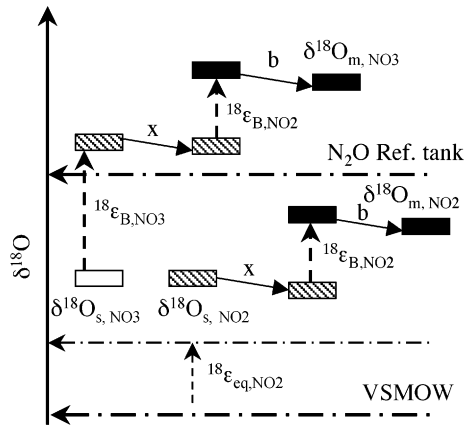

Figure 1. Oxygen isotopic systematics during nitrate and nitrite reduction to N_2O. Open boxes represent nitrate, hatched boxes represent nitrite, and filled boxes represent N_2O. When nitrate is reduced to N_2O, 2.5 of the original 3 oxygen atoms are lost as water and 0.5 is transferred to the N_2O pool. This represents a "branching" of oxygen atoms during the reduction of nitrate and nitrite to N_2O. Isotopic fractionation occurs at these branch points, such that ^{16}O is preferentially lost as water and ^{18}O is preferentially transferred to the subsequent NO_x pool. We define ${}^{18}\epsilon_{B,NO_3}$ as the branching isotope effect during nitrate reduction to nitrite and ${}^{18}\epsilon_{B,NO_2}$ as the branching isotope effect during nitrite reduction to N_2O. Oxygen isotopic exchange between nitrite and water is represented by *x*, and N_2O blank is represented by *b*. In this example, *b* has the effect of lowering $\delta^{18}O_m$ because of their relative $\delta^{18}O$ values. Dot-dashed lines represent the relative $\delta^{18}O$ of the N_2O reference tank and VSMOW, as well as nitrite in equilibrium with VSMOW offset from VSMOW by ${}^{18}\epsilon_{eq,NO_2}$, the equilibrium isotope effect between nitrite and water.

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nitrate conversion to N_2O. In this work, the amount of exchange (x_{NO_2} in eq 1) that occurs during reduction of nitrite to N_2O, the isotopic fractionation between nitrite and N_2O ($^{18}\epsilon_{B,NO_2}$), and the equilibrium isotope effect between nitrite and water ($^{18}\epsilon_{eq,NO_2}$) were determined from analyses using the denitrifier method on independently calibrated nitrite salts.

The amount of exchange (x_{NO_2}) was measured using methods similar to those used to measure isotopic exchange during nitrate conversion to N_2O (x_{NO_3}).[29] Standard solutions (20 μmol L^{-1}) of the calibrated nitrite salts N23, N7373, and N10219 were freshly prepared in deionized water. These solutions were injected into prepared vials of *P. aureofaciens* using the procedure outlined above. In addition, 1 mL of ^{18}O-enriched water ($\delta^{18}O = 255‰$) was added directly to a subset of the sample vials before the injection of calibrated nitrite solutions. Isotopic exchange during the conversion was then calculated from the slope of the $\delta^{18}O_{H_2O}$ versus $\delta^{18}O_m$ regression for each nitrite standard, each of which should provide an independent estimate of the exchange. Nitrate reference materials USGS32, USGS34, and USGS35 were also analyzed in parallel with and without ^{18}O-enriched water for comparison. This procedure was carried out independently on three separate days.

Storage of Nitrite in Freshwater. Solutions of two calibrated nitrite salts, N23 and N7373, were used to test exchange of oxygen atoms between nitrite and water during storage in freshwater at various pH values, temperatures, and nitrite concentrations. First, ^{18}O-enriched water ($\delta^{18}O = +199‰$) was prepared gravimetrically by bringing 35.33 g of ^{18}O-enriched water (0.803 at. % ^{18}O; Icon Stable Isotopes) up to 500 g in DI water. This water was then separated into four aliquots and adjusted to pH values of 6, 8, 10, and 12 by titration with KOH. Concentrated stock solutions (20 mmol L^{-1}) of N23 and N7373 were made up in DI water and added to the pH-adjusted ^{18}O-enriched waters to achieve the desired nitrite concentrations (20 μmol L^{-1}, 200 μmol L^{-1}, or 2 mmol L^{-1}). The pH 6, 8, and 12 waters were incubated at 4 °C. The pH 10 water was subdivided into aliquots to be stored at +22, +4, and −20 °C. The frozen samples were stored in individual aliquots (5 mL) to allow for a time course of analyses while avoiding multiple freeze/thaw cycles. The effect of nitrite concentration on oxygen isotopic exchange was also tested using 20 μmol L^{-1}, 200 μmol L^{-1}, and 2 mmol L^{-1} solutions of both N23 and N7373, prepared at pH 10 and stored at +4 °C.

Samples (16 in all) were analyzed in duplicate on the day of preparation and approximately weekly for up to 2 months. On each day of analysis, stored samples were thawed, subsampled, or diluted to 20 μmol L^{-1}, as needed, and analyzed using the denitrifier method, along with freshly prepared 20 μmol L^{-1} N23 and N7373 solutions in DI water, which served as the primary $\delta^{18}O$ standards. Nitrate isotopic reference materials USGS32, USGS34, and USGS35 were also analyzed in each batch of samples in order to compare the $\delta^{18}O$ normalization scale factors and offsets for nitrate and nitrite.

Storage of Nitrite in Seawater. Storage of nitrite in seawater was tested over a narrower pH range, based on the results obtained in freshwater. Calibrated nitrite salts N23, N7373, and N10219 were added individually to replicate 50-mL aliquots of artificial seawater at final concentrations of 20 μmol L^{-1}. To one 50-mL aliquot of each nitrite material, 1 mL of 6 N NaOH was added to raise the pH to 12; the other aliquot was maintained at pH 7.9. Aliquots of each solution were analyzed immediately using the denitrifier method, with the remainder of each solution divided into two aliquots to be stored either at +21 or −20 °C. Aliquots of each solution were again analyzed by the denitrifier method after 2 and 3 weeks of storage. To test whether elevated pH would interfere with isotopic analysis by the denitrifier method, samples were analyzed with and without pH readjustment. To readjust pH 12 samples to pH 8 using HCl, 75 μL of 6 N HCl was added to 4-mL aliquots just prior to analysis. To readjust samples to pH 8 using acetic acid, 37.5 μL of 6 N acetic acid was added to 2-mL aliquots prior to analysis. All analyses were made in duplicate on a given day and normalized to fresh N23, N7373, and N10219 standards.

Optimal seawater storage temperature was tested in a separate experiment. Aliquots of N7373 and N10219 were prepared in glass vials (30 nmol of NO_2^- in 3 mL of autoclaved artificial seawater) four times, staggered over the course of 35 days and stored in duplicate at +21, +4, −20, and −80 °C. The artificial seawater was made from DI water containing 423 mmol L^{-1} NaCl, 9 mmol L^{-1} KCl, 9.27 mmol L^{-1} $CaCl_2$, 22.94 mmol L^{-1} $MgCl_2$, 25.50 mmol L^{-1} $MgSO_4$, and 2.14 mmol L^{-1} $NaHCO_3$ and had a pH of 7.8. After 35 days, all aliquots were removed from storage, analyzed isotopically, and normalized to fresh nitrite solutions in a single run using the azide method.[28]

Nitrite−Water Equilibrium Isotope Effect. Eight incubations were set up to measure the nitrite/water equilibrium isotope effect at room temperature (+21 °C) and natural pH (7.9). Deionized water and aged natural seawater from Vineyard Sound, MA, were both autoclaved and prepared in duplicate for equilibration experiments as follows: (1) 250 mL of DI water buffered to pH 7.9 with 100 μmol L^{-1} potassium phosphate buffer ($\delta^{18}O_{H_2O} \approx -6‰$), (2) 250 mL of phosphate-buffered DI water amended with 260 μL of 10% ^{18}O water (Cambridge Isotopes) ($\delta^{18}O_{H_2O} \approx +46‰$), (3) 250 mL of seawater ($\delta^{18}O_{H_2O} \approx -1‰$), and (4) 250 mL of seawater amended with 260 μL of 10% ^{18}O water (Cambridge Isotopes) ($\delta^{18}O_{H_2O} \approx +52‰$). Sodium nitrite salts N7373 and N10219 were added individually to each of the four water samples to achieve a final nitrite concentration of 20 μmol L^{-1} in each solution. In these experiments, the nitrite provided a fraction of less than 10^{-6} of the total oxygen atoms ($2NO_2^- + H_2O$), and the equilibrium $\delta^{18}O$ value of nitrite should depend on the $\delta^{18}O$ of the water and the equilibrium isotope effect but not the initial $\delta^{18}O$ of the nitrite salt. Nitrite in each incubation was analyzed for $\delta^{18}O$ in duplicate using the denitrifier method immediately after the solutions were made up and approximately weekly after that for a period of ~8 months, or until the $\delta^{18}O$ values of the nitrite ceased to change. The reaction of multiple nitrite salts having different $\delta^{18}O$ values with a single water source (fresh or sea) provides important evidence for exchange reversibility as well as independent estimates of the equilibrium isotope effect under otherwise identical conditions.

Additional experiments were performed by adding aliquots of a single reagent $NaNO_2$ to fresh meteoric waters with two different $\delta^{18}O$ values: Virginia DI water at −6.0‰ and Antarctic snowmelt water at −49.7‰. Each mixture, containing 50 g of water with 1 mol L^{-1} $NaNO_2$, was acidified with 5 drops of concentrated HCl (pH 4−5) and stored in glass bottles with evaporation-resistant

Table 1. Isotopic Data for Materials Used in This Study

material	ID	$\delta^{15}N$ (‰ vs air)	$\delta^{18}O$ (‰ vs VSMOW)	ref
KNO_3	USGS34	−1.8	−27.9	32
KNO_3	IAEA-N3	+4.7	+25.6	32
KNO_3	RSIL-N11	+3.6	+26.7	32
KNO_3	USGS32	+180.0	+25.7	32
$NaNO_3$	USGS35	+2.7	+57.5	32
KNO_2	RSIL-N20	−79.6	+4.5	this study[a]
$NaNO_2$	RSIL-N7373	−79.6	+4.5	this study[a]
$NaNO_2$	RSIL-N23	+3.7	+11.4	this study[a]
$NaNO_2$	RSIL-N10219	+2.8	+88.5	this study[a]
N_2O	RSIL-N51	+0.7	+42.9	this study[b]
N_2O	RSIL-R6[c]	−0.1	+42.4	this study[d]

[a] $\delta^{15}N$ from off-line combustion and dual-inlet MS; $\delta^{18}O$ from CO-CFIRMS. [b] $\delta^{15}N$ and $\delta^{18}O$ from CFIRMS analysis against a N_2O reference gas (NY-WL) calibrated previously by Yoshinari et al.[45] [c] RSIL-R6 served as the working N_2O reference gas for the denitrifier method N_2O analyses. [d] $\delta^{15}N$ and $\delta^{18}O$ from dual-inlet MS against N51.

Polyseal caps. In this experiment, oxygen atoms in nitrite represented a significant amount (3.6%) of the total oxygen atoms. Each mixture was subsampled several times over periods of weeks to months, treated with KOH to bring the pH to >11 to prevent further isotopic exchange, and then analyzed for $\delta^{18}O$ by the denitrifier method with N7373 and N10219 as reference materials.

RESULTS AND DISCUSSION

Calibrated Nitrite Salts. Commercially available nitrite salts analyzed by CO-CFIRMS as part of this study have $\delta^{18}O$ values from about +5 to +15‰ on the VSMOW scale (Table 1), while the $\delta^{15}N$ values of these nitrite salts ranged from −79.6 to +3.7‰ versus AIR. Broad ranges of both nitrogen and oxygen isotopic values are needed to effectively calibrate isotopic analyses of nitrite samples and to compare the results from different analytical methods. In order to normalize oxygen isotopes in nitrite on a broader scale, a nitrite salt with a significantly higher $\delta^{18}O$ value was prepared by equilibration of nitrite with ^{18}O-enriched water. The resulting nitrite salt, designated N10219 had a $\delta^{18}O$ value of +88.5‰ versus VSMOW. This high $\delta^{18}O$ nitrite material offers a marked extension of the $\delta^{18}O$ calibration scale for nitrite and permits accurate determination of the nitrite scale factor and the branching isotope effect for nitrite conversion to N_2O (see below).

When analyzed by the denitrifier method with *P. aureofaciens* and calibrated against the nitrate reference materials RSIL-N11 and USGS32, the $\delta^{15}N$ values of N20 and N23 were −79.4 ± 0.3 and +3.8 ± 0.2‰, respectively, indistinguishable from the values given in Table 1 from the offline method. The apparent $\delta^{15}N$ value determined by the denitrifier method for N10219 (+2.5 ± 0.2‰) consistently appeared to be slightly lower than the value from the offline method (+2.8‰). This difference is most likely due to the non-mass-dependent ^{17}O content of the Icon water used to prepare ^{18}O-enriched water for the equilibration. This water, while enriched in ^{18}O, is not proportionally enriched in ^{17}O. Thus, the ^{17}O contribution to the m/z 45 signal calculated from the ^{18}O signature in the produced N_2O is an overestimate of the true ^{17}O correction and artificially lowers the calculated $^{15}N/^{14}N$ ratio of the nitrite. This is in contrast to atmospheric nitrates that have *more* ^{17}O than expected from the ^{18}O content, which tends to yield artificially high nitrate $\delta^{15}N$ values via the denitrifier method.[27,36]

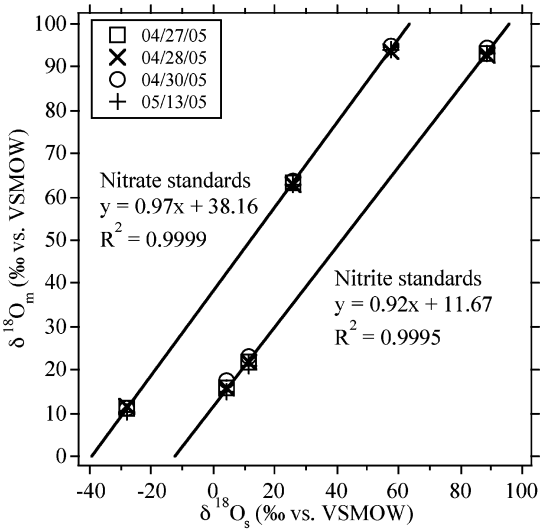

Figure 2. Calibration curves for nitrate and nitrite $\delta^{18}O$ analysis. Measured $\delta^{18}O$ values of N_2O produced from nitrate and nitrite by the denitrifier method with *P. aureofaciens* ($\delta^{18}O_m$) are plotted against the $\delta^{18}O$ values of the same nitrate and nitrite samples analyzed by quantitative online high-temperature conversion to CO (CO-CFIRMS). Data are shown for four separate days of nitrate and nitrite analysis by the denitrifier method. The vertical offset between the calibration curves is largely dictated by $^{18}\epsilon_{B,NO_3}$, the branching fractionation between nitrate and nitrite.

Oxygen Isotopic Analysis of Nitrite by the Denitrifier Method. In practice, calibration curves for nitrite $\delta^{15}N$ and $\delta^{18}O$ analyses are generated from the calibrated nitrite materials analyzed periodically over the course of a sample run. In our laboratory, a suite of reference materials is analyzed after every nine unknown samples. A calibration curve thus generated for each batch of samples is used to calculate the $\delta^{18}O_s$ (‰ vs VSMOW) of the unknowns from their measured ($\delta^{18}O_m$) values, according to eq 1 and as shown in Figure 2. Calibration curves for $\delta^{15}N$ analysis primarily offer a blank correction.[27] In testing the performance of the denitrifier method for oxygen isotopic analysis of nitrite, both nitrate and nitrite were analyzed in parallel for purposes of comparison and cross-calibration (Figure 2). $\delta^{18}O_m$, the measured $\delta^{18}O$ of the N_2O produced from the nitrite (or nitrate), calibrated provisionally with respect to VSMOW via the N_2O reference tank, is plotted versus $\delta^{18}O_s$, or the "true" value of the appropriate nitrite (or nitrate) sample determined independently by online CO-CFIRMS analysis, as described above. The linear regression of these two variables should yield the scaling factor for oxygen isotopic analysis of nitrite (or nitrate) by the denitrifier method. Figure 2 illustrates these calibrations from four separate days of paired nitrate and nitrite analysis. Most clearly evident is the isotopic offset between the nitrate and nitrite calibration curves imposed by the branching fractionation between nitrate and nitrite, $^{18}\epsilon_{B,NO_3}$. Under typical conditions, this term dominates the difference in $\delta^{18}O_m$ for nitrate and nitrite with the same $\delta^{18}O_s$ (eqs 1 and 2).

In addition to the offset between nitrate and nitrite calibration curves, the scale factor for nitrite is contracted by ~5% relative to the nitrate scale factor. The slopes for the nitrate calibration curves on these 4 days of analyses averaged 0.974 ± 0.008 (95% confidence interval), and for the nitrite calibration curves, the

average slope was 0.923 ± 0.014 (95% confidence interval). This difference in calibration slope could reflect a higher blank size for nitrite relative to nitrate or a greater amount of oxygen isotopic exchange prior to or during nitrite conversion to N_2O. The percent oxygen isotopic exchange during conversion of nitrate to N_2O by *P. aureofaciens* in the denitrifier method ranges from 2 to 5%.[29] Here we quantified the amount of oxygen isotopic exchange catalyzed by *P. aureofaciens* during nitrite conversion to N_2O by analyzing nitrite materials N23, N7373, and N10219 with and without the addition of ^{18}O-enriched water to the sample vials. If isotopic exchange occurs, this water will be incorporated into N_2O, causing a proportional increase in $\delta^{18}O$ of N_2O for a given increase in $\delta^{18}O_{H_2O}$. Oxygen isotopic exchange during the reaction was calculated from the blank-corrected slope of the linear regression of $\delta^{18}O_{m,NO_2}$ versus $\delta^{18}O_{H_2O}$, from analyses carried out on three separate days. The apparent incorporation of water during nitrite reduction to N_2O (2.6 ± 1.3%) was indistinguishable from the incorporation during nitrate reduction to N_2O (2.6 ± 0.9%). These observations are consistent with the hypothesis that much of the oxygen isotopic exchange during nitrate reduction to N_2O occurs at the nitrite reduction step and suggest that large differences in oxygen isotopic exchange during conversion of nitrate and nitrite to N_2O are unlikely to cause the difference in calibration slopes. Exchange of oxygen isotopes with water in nitrite salts prior to analysis could also contribute to the observed trends. However, analyses of these nitrite materials over the course of several months, including within 1 day of the CO-CFIRMS analysis, show that the oxygen isotopic calibration slopes were consistent and did not decay with time, as would be expected if exchange were taking place in the salts. Together with the observation that oxygen isotopic exchange during nitrate and nitrite conversion to N_2O are indistinguishable, this consistency with time requires the consideration of higher blanks for the nitrite standards relative to the nitrate standards.

The primary blank associated with the denitrifier method is thought to be residual N_2O in the bacterial medium.[27] This N_2O blank is measured with each set of samples and is routinely ~0.15 nmol, or 1.5% of a 10-nmol sample. However, if calibrated nitrate and nitrite samples are run with the same batch of bacteria, as were the data presented in Figure 2, this blank is unlikely to be different for nitrate and nitrite samples. If a nitrate blank is present in the calibrated nitrite salts themselves, however, this could cause an offset between the CO-CFIRMS-derived values and the bacterially derived values, with the overall effect depending both on the amount and $\delta^{18}O$ of any potential nitrate contaminant present in each nitrite salt. Independent analyses of the nitrite salts by ion chromatography yielded NO_3^-/NO_2^- molar ratios of 0.0016, 0.018, and 0.0067 for N23, N7373, and N10219, respectively, which are too small to explain the calibration slope differences between nitrate and nitrite standards. While a satisfactory explanation for the difference in calibration slope for nitrate and nitrite isotopic analyses cannot be given at this time, the consistencies of the slope and offset provide a measure of confidence in the behavior of nitrite oxygen isotopic analysis using the denitrifier method.

Implications for Oxygen Isotopic Analysis of Mixed Samples Containing Nitrate and Nitrite. Environmental samples may contain mixtures of nitrate and nitrite. Separate analyses of these species may now be possible with methods developed for selective analysis of nitrite[28] and nitrate[38] or by subtraction of nitrite from the $NO_3^- + NO_2^-$ pool (Casciotti, unpublished). One of the important implications of the results summarized above, however, is that routine O isotopic analysis of nitrate by the denitrifier method may give erroneous results if nitrite is present in the sample and is not properly accounted for. Because of the difference between the branching isotope effects, N_2O produced from nitrate by *P. aureofaciens* would have a higher apparent $\delta^{18}O$ than N_2O produced from nitrite with the same initial $\delta^{18}O$ (Figure 1). As a result, a calibration curve based on analyses of nitrate isotopic reference materials will return a value of $\delta^{18}O$ for the nitrite component of a mixture that is as much as 25−30‰ too low (Figure 2). Within the typical uncertainty of nitrate $\delta^{18}O$ measurements (0.5‰), the presence of nitrite as 2% or more of the total nitrate plus nitrite in a sample would give a detectable error in the apparent nitrate $\delta^{18}O$. The effect can be modified by the relative $\delta^{18}O$ values of nitrate and nitrite (larger offset if $\delta^{18}O_{NO_3} > \delta^{18}O_{NO_2}$, smaller offset if $\delta^{18}O_{NO_3} < \delta^{18}O_{NO_2}$). In many environments, nitrate dominates the pool of oxidized nitrogen, but given the potential errors associated with even a small amount of unrecognized nitrite, it is important to know whether nitrite is a significant component of the analyte in a sample.

Nitrite Storage in Freshwater. Nitrite oxygen atoms can be exchanged rapidly with water both biologically and abiologically at low pH.[28,30,32,39] However, little is known about the rate of oxygen exchange between nitrite and water at near-neutral pH. Preservation of the oxygen isotopic composition of a sample from the time it is collected to the time it is analyzed is important for the proper interpretation of nitrite oxygen isotopes in an environmental context. We therefore undertook a systematic evaluation of storage conditions required to maintain oxygen isotopic integrity in nitrite samples and reference materials. Solutions of nitrite salts N23 and N7373 were used in storage experiments to establish conditions under which abiotic oxygen isotopic exchange could be minimized for freshwater samples. The use of two different $NaNO_2$ salts that should behave similarly in solution served to replicate the findings and bolster the recommendations.

The effects of systematic variations in temperature (−20, +4, and +22 °C), pH (6, 8, 10, 12), and nitrite concentration (20 μmol L^{-1}, 200 μmol L^{-1}, and 2 mmol L^{-1}) on nitrite storage are shown in Figure 3. For each treatment the percent oxygen isotopic exchange was calculated from the shift of $\delta^{18}O_{s,NO_2}$ toward full equilibrium, given by $(\delta^{18}O_{s,NO_2} - \delta^{18}O_{s,NO_2 0})/(\delta^{18}O_{H_2O} + {}^{18}\epsilon_{eq,NO_2} - \delta^{18}O_{s,NO_2 0})$, where $\delta^{18}O_{s,NO_2,0}$ denotes the initial δO_2 of the nitrite material. The final equilibrium value for nitrite is given by $\delta^{18}O_{H_2O} + {}^{18}\epsilon_{eq,NO_2}$, where ${}^{18}\epsilon_{eq,NO_2}$ is the equilibrium isotope effect between nitrite and water. For both N23 and N7373, storage at pH 6, 8, and 10 at +4 °C resulted in substantial exchange of oxygen isotopes with water. Within 3 weeks, samples stored at pH 6 and 8 had exchanged 10−30% of nitrite oxygen isotopes with water. Samples stored at pH 10 showed marginally better results, with nitrite oxygen isotopes exchanging less than 5% in the first 3 weeks and then increasing to nearly 30% after 7 weeks. In contrast, samples stored at pH 12 consistently showed less than 0.5% exchange after 8 weeks. Freezing did not routinely improve

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(39) Andersson, K. K.; Philson, S. B.; Hooper, A. B. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 5871−5875.

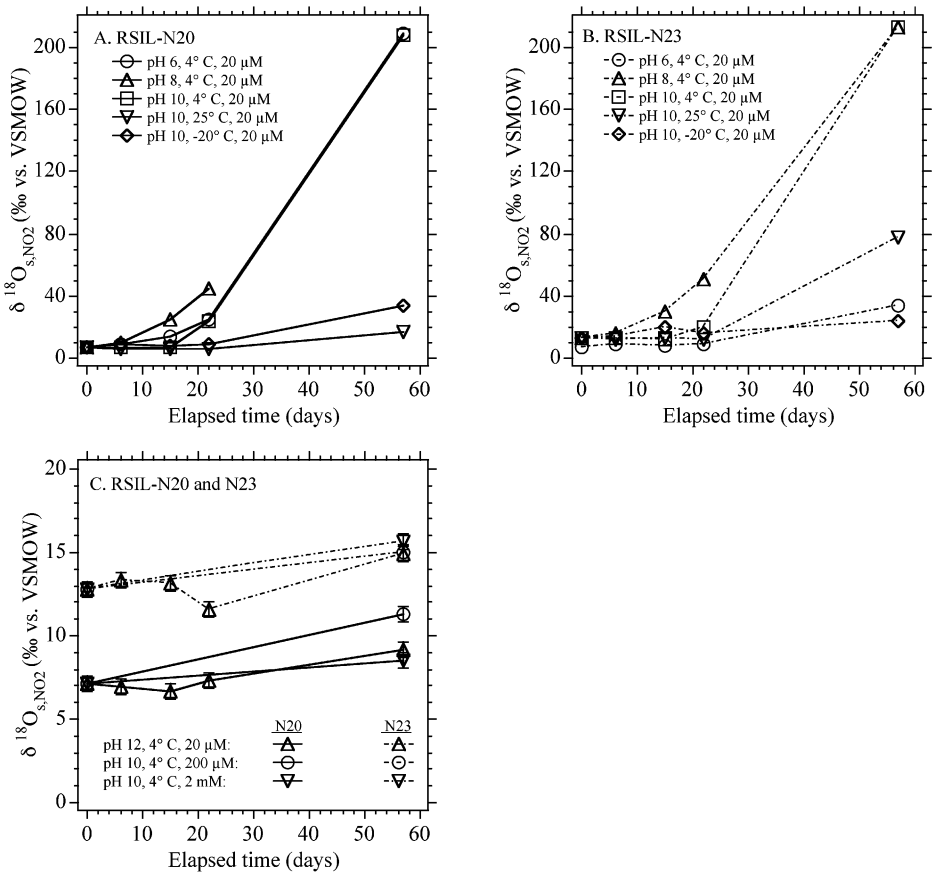

Figure 3. Isotopic effects of nitrite storage in freshwater. Nitrite samples N20 and N23 were stored in freshwater with a $\delta^{18}O_{H_2O}$ value of +199‰ at systematically varied temperatures, concentrations, and pH values for a period of ~8 weeks. Measured $\delta^{18}O$ values of stored nitrite solutions were normalized to freshly prepared nitrite solutions analyzed in the same run on each day of analysis. Results are divided into high-exchange (unsuccessful) treatments (A, B) and low-exchange (more successful) treatments (C). The most successful treatments comprised storage at pH 12 at 4 °C for solutions of 20 μmol L^{-1} nitrite and at pH 10 for higher concentrations of nitrite. Error bars in (A) and (B) are smaller than the symbols.

preservation in freshwater at pH 10. For example, N7373 stored at pH 10 showed less exchange at room temperature than with refrigeration or freezing, while for N23, freezing outperformed refrigeration or room-temperature storage. The 200 μmol L^{-1} and 2 mmol L^{-1} solutions of N7373 and N23 (both at pH 10) showed low levels of exchange, similar to the 20 μmol L^{-1} pH 12 solutions. Although higher concentrations can be used for storing nitrite standards, most natural samples contain nitrite at concentrations less than 20 μmol L^{-1} and storage conditions that require elevated concentrations of nitrite are not practical for preservation. From these results, it is clear that storage at −20 °C of samples with pH of 10 or less does not guarantee maintenance of nitrite oxygen isotopes in freshwater. The recommended storage procedure for nitrite in freshwater is thus to adjust the pH to 12 and store refrigerated or frozen. Room-temperature storage at pH 12 may be sufficient, but was not tested here.

Nitrite Storage in Seawater. The conditions for storage of nitrite in seawater were not examined as thoroughly as freshwater storage conditions in this study. However, short-term experiments showed that basic preservation may also be a good approach for storage of nitrite in seawater. Nitrite stored in artificial seawater at pH 8 equilibrated 36% over two weeks of storage at room temperature and 53% over three weeks. Freezing samples gained a slight advantage, with 27% equilibration after two weeks of storage at −20 °C and 38% equilibration after three weeks. Although a small blank (<0.3 nmol) was involved in addition of NaOH, nitrite solutions adjusted to pH 12 retained their oxygen isotopic signatures considerably better after three weeks of storage than pH 8 solutions, at both room temperature and −20 °C. Nitrite samples that were preserved in artificial seawater at pH 12 for three weeks exchanged less than 2−3% of their oxygen isotopes with water (Figure 4A). An important result from these experiments, however, was that neutralization of base-preserved seawater samples using 6 N HCl caused immediate equilibration of nitrite with water, despite maintenance of the pH above 8.0 (Figure 4A). Neutralization of the pH 12 samples was not required for isotopic analysis by the denitrifier method for the small volumes (1 mL) analyzed here, as fresh nitrite samples analyzed with and without base addition yielded indistinguishable $\delta^{18}O$ results (not shown). However, larger volume samples, which would be required to analyze nitrite isotopes at lower concentrations, may overwhelm the buffering capacity of the bacterial medium. To analyze such samples, alternative means of restoring basic samples to near-neutral pH are being investigated, such as neutralization with a weaker acid, such as acetic acid (Figure 4A).

To further examine storage of nitrite in seawater, storage temperature was tested in an experiment lasting 35 days in autoclaved artificial seawater. $\delta^{18}O$ values of nitrite in solutions

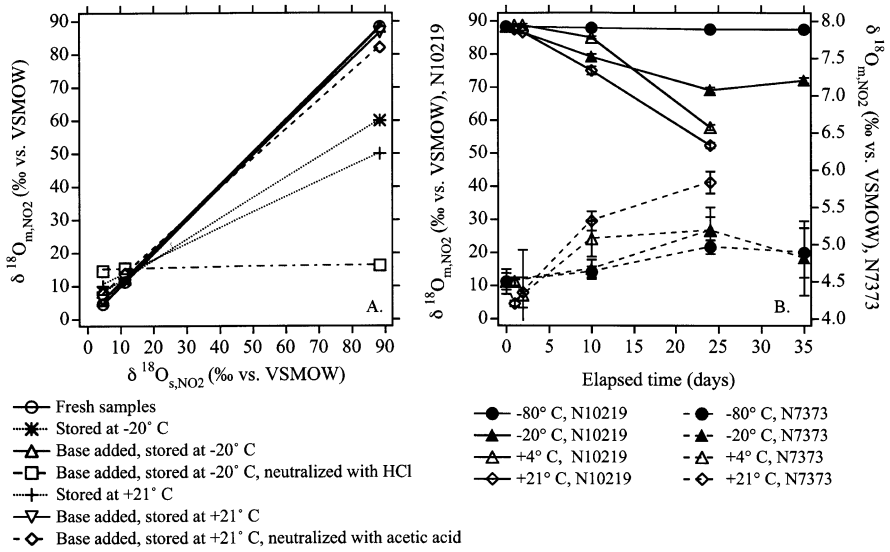

Figure 4. Isotopic effects of nitrite storage in artificial seawater tested at various pH values (A) and temperatures (B). In (A), three nitrite samples with different initial $\delta^{18}O_{s,NO_2}$ (N23, N7373, and N10219) were stored for three weeks at +21 and −20 °C at pH 7.9 and pH 12. Aliquots of samples stored at pH 12 were analyzed for $\delta^{18}O_{NO_2}$ with and without neutralization using HCl (or acetic acid). Isotopic results are based on duplicate analyses normalized to VSMOW using fresh nitrite samples run in parallel on each day of isotopic analysis. Error bars in (A) are smaller than the symbols. As with storage of nitrite in freshwater, basic preservation improved retention of nitrite oxygen isotopic values in seawater. In (B), two nitrite samples (N7373 and N10219) were stored at pH 7.9 at four temperatures (+21, +4, −20, and −80 °C) for 5 weeks. Subsamples taken over that time period were analyzed using the denitrifier method and normalized to VSMOW using fresh nitrite samples analyzed in parallel. Best results were obtained from storage at −80 °C and preservation with hydroxide at pH 12.

made from N7373 and N10219 were analyzed four times during storage at temperatures of −80, −20, +4, and +21 °C (Figure 4B). Both nitrites stored in artificial seawater at near-neutral pH (7.8) showed measurable equilibration (given in %) after 24 days of storage at room temperature (30−45%) and +4 (15−38%), −20 (15−20%), and −80 °C (1−10%). In these experiments, storage at −80 °C maintained the $\delta^{18}O$ of the nitrite in solution closest to the original value. At this point, it appears that filtering and freezing samples at −80 °C can be used to preserve the isotopic composition of nitrite in seawater samples prior to analysis. Hydroxide preservation also may give good results, but neutralization with HCl prior to analysis is not recommended. In any case, caution should be taken in interpreting nitrite oxygen isotopic values from seawater unless changes during storage can be definitively ruled out (i.e., by internal standard additions). Additional tests are underway to determine whether immediate conversion to N_2O by sodium azide[28] and storage as N_2O will represent a better storage solution for nitrite in seawater.

Nitrite−Water Equilibrium Isotope Effect. The rapid exchange of oxygen isotopes between nitrite and water observed during storage at near-neutral pH stimulated an investigation of the isotopic offset between nitrite and water at equilibrium. This isotope effect has not previously been discussed, although it is likely to be important for understanding the oxygen isotopic signatures of nitrite and nitrate in the environment. At equilibrium, oxygen isotopes are not distributed equally between nitrite and water. This unequal distribution is the expression of an equilibrium isotope effect, causing nitrite to be preferentially enriched in ^{18}O relative to the water with which it is in equilibrium. In this study, two separate approaches for measuring equilibrium isotope effects between nitrite and water were followed.

In the first, nitrite salts with different initial $\delta^{18}O$ values (N7373 and N10219) were each equilibrated with four different water sources: DI water (pH 7.9) or seawater (pH 7.95), each adjusted to two different $\delta^{18}O$ values with ^{18}O-enriched water (Table 2). Equilibrium was achieved when the two nitrite solutions had reached the same $\delta^{18}O$ value (or ceased to change $\delta^{18}O$ value) in a given water sample, which took more than 8 months for both seawater and freshwater incubations at 21 °C. For N7373, equilibration with water raised the $\delta^{18}O$ of the nitrite in each treatment, and for N10219, equilibration with water lowered the $\delta^{18}O$ of the nitrite in each treatment. From the $\delta^{18}O$ of the water and $\delta^{18}O$ of each nitrite sample at equilibrium, the equilibrium isotope effects were calculated as follows:

$$^{18}\epsilon_{eq,NO_2} = (^{18}R_{NO_2}/^{18}R_{H_2O} - 1) = (\delta^{18}O_{NO_2} - \delta^{18}O_{H_2O})/(\delta^{18}O_{H_2O} + 1) \quad (3)$$

with $\delta^{18}O$ values reported versus VSMOW (see Terms and Definitions). The equilibration of two separate nitrite materials in a given water sample, with and without moderate ^{18}O-enrichment of the water, provided multiple levels of experimental replication in the measurement of $^{18}\epsilon_{eq,NO_2}$ in seawater and freshwater. Treatments in seawater yielded values of $^{18}\epsilon_{eq,NO_2}$ around +14.0‰, with an uncertainty of ∼0.5‰ based on analytical accuracy and precision of the equilibrated nitrite $\delta^{18}O$ values. Freshwater treatments yielded an equilibrium isotope effect around +14.4‰, again with an uncertainty of 0.5‰ (Table 2).

In another set of acidified (pH 4−5) freshwater experiments, a single $NaNO_2$ reagent with an initial $\delta^{18}O$ of +6.3‰ was equilibrated with two isotopically different waters at 22 °C. In each case, the $\delta^{18}O$ value of the nitrite changed and reached a steady value within the analytical uncertainty after 2−3 months. In water with an initial $\delta^{18}O = -6.0$‰, the final $\delta^{18}O$ value of the nitrite

Table 2. Nitrite−Water Equilibrium Isotope Effects

materials[a]	temp (°C)	$\delta^{18}O$-H_2O (‰ vs VSMOW)[b]	$\delta^{18}O$-NO_2^- (‰ vs VSMOW)[c]	$^{18}\epsilon_{eq,NO_2}$ (‰)
N-7373 in FW	21	−6.0	+8.3	+14.4 ± 0.5
N-10219 in FW	21	−6.0	+8.4	+14.5 ± 0.5
N-7373 in FW + ^{18}O	21	+45.6	+60.6	+14.3 ± 0.5
N-10219 in FW + ^{18}O	21	+46.8	+61.9	+14.4 ± 0.5
N-7373 in SW	21	−1.0	+13.0	+14.0 ± 0.5
N-10219 in SW	21	−1.1	+12.8	+13.9 ± 0.5
N-7373 in SW + ^{18}O	21	+52.5	+67.1	+13.9 ± 0.5
N-10219 in SW + ^{18}O	21	+51.5	+66.2	+14.0 ± 0.5
$NaNO_2$ in DW	22	−6.0 (− 6.0)[d]	+7.3 ± 0.6	+13.4 ± 1.0
$NaNO_2$ in DW	22	−49.7 (−48.2)[d]	−35.2 ± 0.8	+13.7 ± 1.0

[a] FW, phosphate-buffered deionized water, see text for details; SW. aged natural seawater, see text for details; DW. deionized water; ^{18}O. Cambridge Isotopes 10% ^{18}O water, see text for details. [b] Precision of $\delta^{18}O$-H_2O analysis is 0.2%. [c] Unless otherwise noted, overall analytical uncertainty for $\delta^{18}O$-NO_2^- analyses is estimated to be 0.5%, although precision on a single day is often better than 0.2%. [d] The first entry is the initial $\delta^{18}O$ value of the water used in the experiment; the second entry (in parentheses) is the final $\delta^{18}O$ value of the water after equilibration, calculated from mass balance and the change in the nitrite $\delta^{18}O$ values.

was +7.3 ± 0.6‰ and the final $\delta^{18}O$ value of the water (calculated by mass balance) was −6.0‰ (Table 2). In water with initial $\delta^{18}O$ = −49.7‰, the final $\delta^{18}O$ value of the nitrite was −35.2 ± 0.8‰ and the estimated final $\delta^{18}O$ of the water was −48.2‰. These results yield equilibrium oxygen isotope effects ($^{18}\epsilon_{eq,NO_2}$) of +13.4 and +13.7‰, respectively, with uncertainties estimated to be ∼1‰ (Table 2).

To summarize these findings, abiotic isotopic exchange of nitrite with either seawater or freshwater will cause the $\delta^{18}O$ value of the nitrite to reach a value that is ∼14‰ higher than that of the coexisting water at equilibrium. Uncertainties in these values may be related to a combination of analytical uncertainties or differences in the speciation of the nitrite or other NO_x compounds in the solutions at different pH values, salinity effects, or other experimental conditions. These results indicate that $^{18}\epsilon_{eq,NO_2}$ is significantly smaller than the equilibrium isotope effect between nitrate and water ($^{18}\epsilon_{eq,NO_3}$) of 23‰ at 22 °C reported for a 1 N HNO_3 solution by Böhlke et al.[33] While the effect of temperature was not explicitly tested here (all experiments were conducted near room temperature), it is expected that $^{18}\epsilon_{eq,NO_2}$ would be dependent on temperature. Independent experiments indicate that $^{18}\epsilon_{eq,NO_2}$ decreases by ∼5‰ as temperature is raised from +4 to +37 °C.[32] This temperature effect is similar in magnitude to that reported for $^{18}\epsilon_{eq,NO_3}$.[33]

Implications. This study has demonstrated critical facets of oxygen isotopic systematics during denitrification, including isotopic fractionation and exchange. These findings are important for the utilization of the denitrifier method in oxygen isotopic analysis of nitrate or nitrite alone or in combination. The procedures described here may be used for producing laboratory supplies of nitrite with varying oxygen isotopic compositions for calibration and experimental purposes. In addition to providing information related to the analysis and calibration of nitrite and nitrate oxygen isotopic ratios, these results have several important implications for understanding oxygen isotopic systematics in natural aquatic environments.

The rapid equilibration of nitrite with both seawater and freshwater at near-neutral pH implies that where decoupling of biological production and consumption of nitrite allows nitrite to accumulate, chemical equilibration may play a significant role in setting the $\delta^{18}O$ of nitrite, as well as downstream products such as nitrate and N_2O. Microbial activities may also enhance nitrite−water equilibration in nature. As seen in both nitrification[29,39,40] and denitrification,[16,21,29,41] processes that cycle nitrite also promote equilibration with water. As a result of isotopic exchange, the $\delta^{18}O$ of nitrite produced by ammonia-oxidizing bacteria will not necessarily evolve to that of the water, but rather to the $\delta^{18}O$ value of the water offset by an equilibrium isotope effect. In the ocean, this could have the effect of enriching nitrite in ^{18}O relative to water, while "washing out" variations in nitrite $\delta^{18}O$ caused by variations in $\delta^{18}O$ of O_2 through the water column. The observation that $\delta^{18}O$ in deep-water nitrate is commonly close to that of seawater,[13,29,42,43] however, may seem surprising in light of this, and additional work is needed to understand the oxygen isotopic systematics of nitrification.

From the data presented here (Figure 2), we can also estimate that, during denitrification, the branching isotope effect between nitrate and nitrite ($^{18}\epsilon_{B,NO_3}$) is 25−30‰, which causes oxygen isotopic enrichment in nitrite relative to the nitrate from which it formed. The magnitude of this effect is in line with that assumed by Sigman and co-workers.[13] While there is substantial kinetic

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isotopic discrimination against ^{18}O during nitrate reduction,[38,44] $^{18}\epsilon_{B,NO_3}$ would thus be expected to at least partially counteract the kinetic fractionation to yield nitrite with $\delta^{18}O$ values that can equal or exceed the $\delta^{18}O$ of nitrate from which it was formed under certain relative rates of nitrite production, consumption, and equilibration with water. We also note that the branching isotope effects demonstrated here and reported previously[29] are likely to contribute to the enrichment of ^{18}O in N_2O in oceanic denitrification zones[11,45,46] due to the nearly 40‰ branching fractionation[28] that enriches N_2O in ^{18}O relative to nitrate (i.e., Figure 1). Furthermore, when N_2O is produced from nitrite by ammonia-oxidizing bacteria utilizing the nitrifier−denitrification pathway,[47−49] a similar branching fractionation may apply. In this case, the N_2O produced would be expected to have a higher $\delta^{18}O$ value than the nitrite from which it is generated. There are still many unknowns in the oxygen isotopic systematics of these processes, including the rates and controls of biologically catalyzed nitrite equilibration with water, but the methods and approaches described here enable some of the measurements needed to address these unknowns.

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