Anal. Chem. **2002,** *74,* 4905−4912

Measurement of the Oxygen Isotopic Composition of Nitrate in Seawater and Freshwater Using the Denitrifier Method

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We report a novel method for measurement of the oxygen isotopic composition ($^{18}O/^{16}O$) of nitrate (NO_3^-) from both seawater and freshwater. The denitrifier method, based on the isotope ratio analysis of nitrous oxide generated from sample nitrate by cultured denitrifying bacteria, has been described elsewhere for its use in nitrogen isotope ratio ($^{15}N/^{14}N$) analysis of nitrate.[1] Here, we address the additional issues associated with $^{18}O/^{16}O$ analysis of nitrate by this approach, which include (1) the oxygen isotopic difference between the nitrate sample and the N_2O analyte due to isotopic fractionation associated with the loss of oxygen atoms from nitrate and (2) the exchange of oxygen atoms with water during the conversion of nitrate to N_2O. Experiments with ^{18}O-labeled water indicate that water exchange contributes less than 10%, and frequently less than 3%, of the oxygen atoms in the N_2O product for *Pseudomonas aureofaciens*. In addition, both oxygen isotope fractionation and oxygen atom exchange are consistent within a given batch of analyses. The analysis of appropriate isotopic reference materials can thus be used to correct the measured $^{18}O/^{16}O$ ratios of samples for both effects. This is the first method tested for $^{18}O/^{16}O$ analysis of nitrate in seawater. Benefits of this method, relative to published freshwater methods, include higher sensitivity (tested down to 10 nmol and 1 μM NO_3^-), lack of interference by other solutes, and ease of sample preparation.

Nitrate is the dominant form of bioavailable nitrogen in the ocean. The $^{15}N/^{14}N$ ratio in nitrate is an important integrative tool for understanding internal cycling (assimilation, remineralization, nitrification) and transport of nitrate[2,3] as well as the balance of inputs and losses of nitrogen in the ocean (nitrogen fixation and denitrification).[2−8] Because the oxygen and nitrogen atoms in nitrate do not record identical aspects of the nitrogen cycle, coupled isotopic measurements have provided complementary information on nitrate in freshwater and terrestrial systems.[9−12] This same complementarity will likely apply in the ocean, but the lack of a method for $^{18}O/^{16}O$ analysis of nitrate in seawater has precluded the use of nitrate oxygen isotopes as a constraint on oceanic nitrogen transformations.

Published methods for $^{18}O/^{16}O$ analysis of nitrate from freshwaters are based on collection of nitrate on anion exchange columns and high-temperature conversion of nitrate to N_2 and CO_2 or CO in the presence of a reduced carbon donor.[13−17] These methods involve relatively complex and labor-intensive purification procedures that are not applicable to seawater samples, which have high Cl^- and SO_4^{2-} concentrations and typically low NO_3^- concentrations. The existence of large and variable blanks from dissolved organic matter is also an issue in the application of combustion-based methods to many sample types.[15] Finally, as described below, uncharacterized oxygen isotope exchange or incorporation of oxygen atoms from other oxides during the high-temperature reaction step can lead to uncertainty in the analyses conducted with off-line combustion methods.

The denitrifier method for $^{15}N/^{14}N$ analysis of nitrate, described by Sigman et al.,[1] is based on bacterial conversion of nitrate to

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10.1021/ac020113w CCC: $22.00
Published on Web 09/07/2002

N_2O and, therefore, can also offer oxygen isotope information. While the conversion of nitrate to N_2O represents a mass balance reaction with respect to nitrogen, this is not the case for oxygen atoms. Only one of six oxygen atoms present in the initial nitrate pool is represented in the N_2O analyte:

$$2NO_3^- \rightarrow 2NO_2^- \rightarrow 2NO \rightarrow N_2O$$

If there is preferential loss of ^{16}O in these reactions, there will be a difference in $^{18}O/^{16}O$ between nitrate and the product N_2O, even with complete conversion of nitrate to N_2O. This oxygen isotopic fractionation must be adequately reproducible for a given batch of analyses to properly correct for this effect.

The second concern in $^{18}O/^{16}O$ analysis of nitrate using the denitrifier method is the exchange of oxygen atoms between the nitrogen oxide intermediates and water, which would introduce non-nitrate oxygen atoms into the N_2O analyte. Enzymatic catalysis of the exchange of water oxygen with nitrite (NO_2^-) and nitric oxide (NO) has been demonstrated in denitrification systems.[18−20] The degree of exchange varies greatly among bacterial strains and may be related to the biochemistry of nitrite reduction.[19] Bacteria possessing the heme-type nitrite reductase (as *Pseudomonas chlororaphis* does[21]) were shown to catalyze a relatively large amount of exchange[19] (39−76%), while *Pseudomonas aureofaciens*, known to possess the copper-type nitrite reductase,[22] was shown to cause relatively little incorporation of oxygen atoms from water into N_2O (6%).[19]

We have established approaches for quantifying and correcting for both fractionation of oxygen isotopes during oxygen atom loss and exchange of oxygen atoms with water that are inherent in the denitrifier method for $^{18}O/^{16}O$ analysis of nitrate. These approaches are described below, as are other tests relevant to the performance of this method, a comparison with previously published freshwater methods, and oxygen isotope ratio measurements of nitrate from an ocean depth profile.

EXPERIMENTAL SECTION

Bacterial Strains and Culture Conditions. *P. aureofaciens* (ATCC 13985 recently reclassified as a strain of *P. chlorophis*), *P. chlororaphis* (provided by J. M. Tiedje), and *Corynebacterium nephridii* (ATCC 11425), are maintained on Tryptic Soy Agar (Difco), amended with 10 mM KNO_3. Starter cultures (5-mL Tryptic Soy Broth) are inoculated from single colonies and grown overnight in sterile test tubes to generate inoculum for working cultures. For *P. aureofaciens* and *C. nephridii*, working cultures are grown in 130-mL batches of Tryptic Soy Broth (Difco) amended with 10 mM KNO_3, 7.5 mM NH_4Cl, and 36 mM KH_2PO_4. These working cultures are inoculated with 0.5 mL of starter culture and grown in butyl rubber-stoppered serum bottles (160-mL capacity) on a reciprocal shaker at room temperature. For *P. chlororaphis*, working cultures are prepared as previously described.[1]

$^{18}O/^{16}O$ Analysis. Sample preparation follows the method of Sigman et al.,[1] which is outlined briefly here. Working cultures grown for 6−10 days are concentrated 10-fold by centrifugation and then split into 2-mL aliquots in 20-mL headspace vials (Alltech #98788). The vials are crimp-sealed with Teflon-backed silicone septa (Alltech #95587) and purged for 3 h with N_2. Samples of dissolved nitrate (10−20 nmol) are then added to the sample vials and are incubated overnight to allow for complete conversion of nitrate to N_2O before the addition of 0.1 mL of 10 N NaOH to stop bacterial activity and scavenge CO_2. A constant sample size (e.g., 20 nmol) is targeted for each batch of samples to simplify the correction of $\delta^{18}O$ values (below). In early method development and application, the N_2O analyte was extracted from sample vials off-line and analyzed using a Finnigan PreCon upstream of a Finnigan MAT 252 IRMS.[1] Subsequently, we adapted a Finnigan GasBench II system for on-line automated extraction and analysis of N_2O (Figure 1), which is described below.

Sample vials are held upright in a custom-made aluminum rack that is immovable relative to the autosampler coordinate system. The exchangeable syringe system of the CTC CombiPAL injection head was modified to allow separate input (He) and outflow (He + sample gas) needles. Gas flow to each needle is regulated separately so that the outflow needle is back-flushed when disengaged from each vial.

The gas flow is directed by an eight-port Valco valve (A4C8WE) that has two positions, referred to as "load" and "inject". While in load mode (Figure 1A), the autosampler needles pierce the vial septum and the N_2O is completely flushed from the sample vial with He carrier gas (25 mL/min). The outflow from the vial passes a Nafion drier (Perma Pure Inc., MD-050-72S-1) and a chemical trap (layered magnesium perchlorate and ascarite) to remove water and CO_2 before entering the Finnigan GasBench II system. The eight-port Valco valve then directs the sample gas through a 95 cm × $^1/_{16}$ in. o.d. stainless steel loop (T1) immersed in liquid N_2 (LN_2) to condense N_2O (as well as any remaining H_2O and CO_2), while the uncondensed gases (He, N_2) vent to the atmosphere. A separate low-flow He carrier (3 mL/min) is directed through a smaller volume trap (T2) made of 56 cm × 0.32 mm i.d. glass capillary, which is not immersed in LN_2 at this point. The low-flow He exiting T2 then flows to the gas chromatograph and mass spectrometer. After 800 s, the eight-port Valco valve is actuated to switch the flow paths into inject mode.

In inject mode (Figure 1B), T1 is brought into the flow path with T2, the gas chromatograph, and the mass spectrometer under the low-flow He. T2 is then lowered into LN_2, and after a 60-s delay to allow T2 to reach LN_2 temperatures, T1 is raised from LN_2. After another 340 s, when transfer of sample gas from T1 to T2 is complete, T2 is raised from LN_2. The sample gas, focused into a smaller volume by the second cryogenic trapping step, is then released under the low-flow He and passes into the gas chromatograph (held at 20 °C), where N_2O is separated from residual CO_2 using a capillary column (25 m × 0.32 mm) lined with Poraplot-Q (Chrompack, 7551). The outflow from the gas chromatograph passes through one final Nafion drier (Perma Pure Inc., TT-020−412) before reaching the "open split" sampled by the mass spectrometer.[23] During inject mode, the chemical trap,

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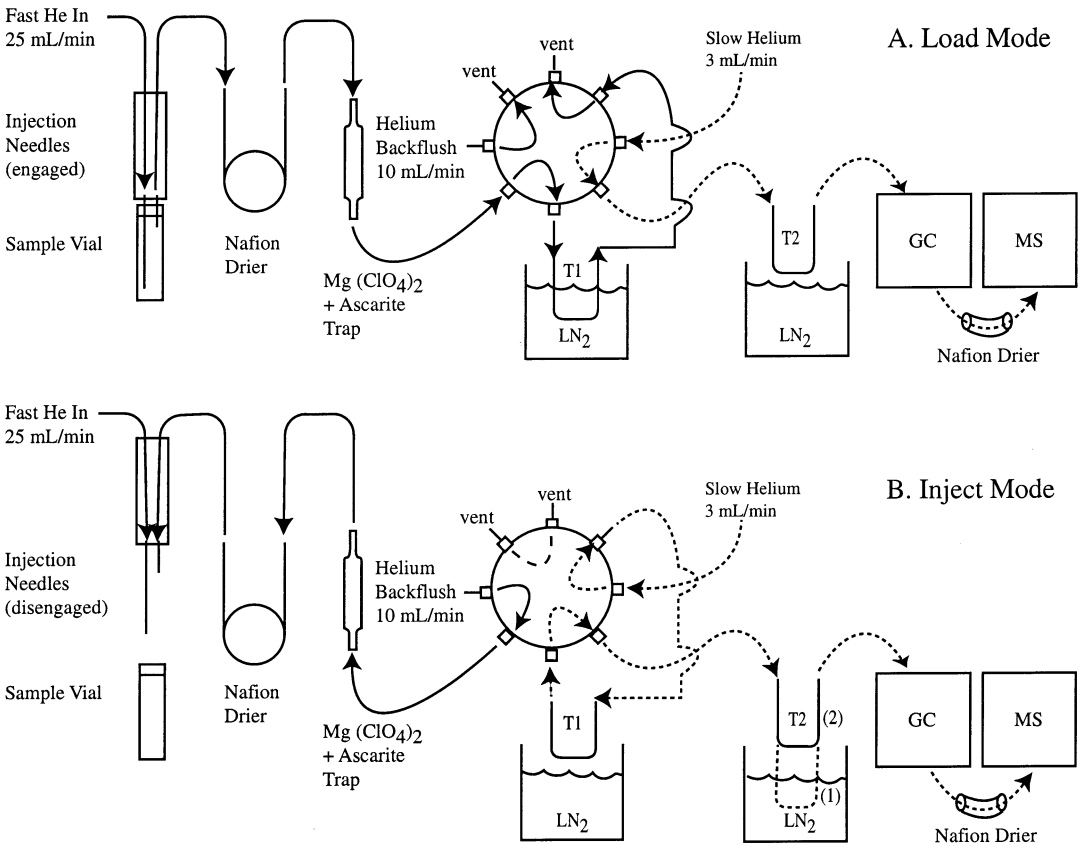

Figure 1. Automated N_2O extraction and $\delta^{18}O$ analysis system. In load mode (A), the N_2O is completely extracted from a sample vial and condensed on the first cryogenic trap (T1). In inject mode, the flow is reversed through T1 and the N_2O is transferred to T2 under the low-flow He carrier (1) and then released (2) to the GC and finally to the mass spectrometer (see text for details).

first Nafion water trap, and autosampler outflow needle are back-flushed with He (Figure 1).

A Finnigan DELTA^{plus} isotope ratio mass spectrometer is used in continuous-flow mode to measure the $m/z = 45/44$ and $46/44$ ratios of each sample. These ratios are measured against pulses of reference N_2O from a gas cylinder, which are introduced by a second open split prior to the arrival of each sample peak at the mass spectrometer.[23] The N_2O reference tank, however, is not our absolute reference. Each set of samples includes replicate analyses of IAEA-NO-3 (subsequently referred to as "N3") that are used to reference nitrate samples to VSMOW. N3 is an internationally distributed KNO_3 reference material with an assigned $\delta^{15}N$ of 4.7‰ versus atmospheric N_2 [24,25] and reported $\delta^{18}O$ of 22.7−25.3‰ versus VSMOW[14,16,17] ($\delta^{18}O = \{[(^{18}O/^{16}O)_{sample}/(^{18}O/^{16}O)_{VSMOW}] - 1\} \times 1000$). For the purposes of this study, we adopt a value of 22.7‰.

Corrections for Oxygen Isotopic Fractionation, Exchange, and Blank. In all methods for $^{18}O/^{16}O$ analysis of nitrate there are potential errors associated with fractionation during sample preparation, exchange of oxygen atoms, and reagent or matrix blanks. Quantification of these errors allows corrections to be applied to measured isotope values to achieve a more accurate result. The $\delta^{18}O$ reached upon complete conversion of nitrate to N_2O depends on isotopic fractionation, exchange, and blank according to the following mass and isotope balance equations:

$$m = s + b \quad (1)$$

$$\delta^{18}O_m m = (\delta^{18}O_s + \epsilon)s(1 - x) + \delta^{18}O_{H_2O}sx + \delta^{18}O_b b \quad (2)$$

where m is the total amount of measured N_2O N in the sample vials, s is the amount of sample nitrate N added, b is the amount of blank N, and x is the fraction of oxygen atoms in the product N_2O that originates from exchange with water during denitrification. $\delta^{18}O_m$ is the measured $\delta^{18}O$ value (vs VSMOW), $\delta^{18}O_s$ is the true $\delta^{18}O$ of the sample nitrate (vs VSMOW), and the oxygen isotopic compositions of the water and blank (vs VSMOW) are given by $\delta^{18}O_{H_2O}$ and $\delta^{18}O_b$, respectively. ϵ is the net isotopic fractionation caused by the removal of oxygen atoms in the reduction of nitrate to N_2O. The use of δ notation, instead of isotope ratios, in eq 2 is an approximation that has little effect on $\delta^{18}O$ values close to our reference material (N3) but imparts errors of up to 1‰ for samples ±50‰ relative to N3. We use this approximate form to simplify the discussion that follows.

The effects of isotopic fractionation, exchange, and blank on $^{18}O/^{16}O$ measurements would ideally be quantified by the analysis of two nitrate reference materials of known $\delta^{18}O$ with each batch of samples. If it can be assumed that exchange, isotope fractionation, and blank size are constant among samples prepared with

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the same culture batch (see below for validation of these assumptions), eq 2 can be rearranged to describe the oxygen isotopic difference between any two samples from the same culture batch:

$$(\delta^{18}O_{s,1} - \delta^{18}O_{s,2}) = \delta^{18}O_{m,1}(s_1 + b)/(s_1(1 - x)) - \delta^{18}O_{m,2}(s_2 + b)/(s_2(1 - x)) \quad (3)$$

where $\delta^{18}O_{m,1}$ and $\delta^{18}O_{m,2}$ are the measured $\delta^{18}O$ values of two samples (vs VSMOW), $\delta^{18}O_{s,1}$ and $\delta^{18}O_{s,2}$ are the true $\delta^{18}O$ values of those two samples (vs VSMOW), s_1 and s_2 are the sizes (nmol of N) of each sample, and b and x are defined as in eqs 1 and 2. Since sample volume is added to achieve uniform N_2O quantities within a batch, the correction factor simplifies to $(s + b)/(s(1 - x))$. From two nitrate reference samples with known $\delta^{18}O_s$ values, the correction factor could then be calculated from the ratio $(\delta^{18}O_{s,1} - \delta^{18}O_{s,2})/(\delta^{18}O_{m,1} - \delta^{18}O_{m,2})$ and applied to correct the $\delta^{18}O_m$ of each sample for the cumulative effect of blank, exchange, and fractionation using the following equation:

$$\delta^{18}O_s = \delta^{18}O_{s,ref} + (\delta^{18}O_m - \delta^{18}O_{m,ref})(s + b)/(s(1 - x)) \quad (4)$$

At this time, lacking two well-constrained oxygen isotope reference materials, estimations of the amounts of exchange and blank must be made independently and then combined to calculate the correction factor. Exchange is calculated for each culture batch from the slope of the regression between $\delta^{18}O_{H_2O}$ and $\delta^{18}O_m$ for N3 in normal and ^{18}O-enriched water ($\delta^{18}O_{H_2O}$ = 325‰), as described below. The size of the culture blank is also measured for each culture batch by running prepared vials to which no sample has been added, as described below. The correction factor $(s + b)/(s(1 - x))$ is then used to calculate the $\delta^{18}O_s$ of each unknown sample using eq 4, incorporating the "true" and measured $\delta^{18}O$ values of the reference, N3 ($\delta^{18}O_{s,ref}$ and $\delta^{18}O_{m,ref}$, respectively).

The present uncertainty for our corrections if the amounts of blank or exchange are not measured with each run, but instead are assumed to be 0.5 nmol blank and 5% exchange, is 0.3 and 0.6‰, respectively for 20-nmol samples that are 20‰ different from N3. The uncertainty is proportionally lower for samples that are more similar in $\delta^{18}O$ to N3.

RESULTS AND DISCUSSION

Bacterial Growth and Culture Conditions. We occasionally obtain *P. aureofaciens* cultures that convert nitrate to nitrite but fail to denitrify nitrite completely to N_2O in the 6−10-day phase of culture preparation. This failure to denitrify is accompanied by low cell density, abnormal cell coloration, and an acrid (bleach-like) odor. We have never obtained a culture batch that fails to completely convert sample nitrate to N_2O for isotopic analysis after growing normally in the initial phase. Thus, this occasional failure of the initial culture preparation is inconvenient but does not lead to uncertainties in the isotope results.

We have investigated the reasons for this occasional failure, which is apparently due to nitrite toxicity. A diverse range of conditions affects whether nitrite consumption occurs in *P. aureofaciens* cultures, including (1) the proportion of headspace to medium volume in culture bottles (which affects the amount of O_2 initially available for growth), (2) the volume and cell density of inoculum, (3) the initial pH and buffering capacity of the medium (which affects the concentration of nitrous acid, HNO_2, the suspected toxin), and (4) prior growth of the culture on lower levels of nitrite (which presumably leads to more reliable induction of the nitrite reductase). However, there is not great consistency in the absolute conditions that allow for nitrite reduction and associated healthy growth of the culture. In the Experimental Section, we described the growth conditions that have optimized growth success in our laboratory.

Oxygen Isotopic Fractionation. The time required for complete conversion of sample nitrate to N_2O was established for *P. aureofaciens* by Sigman et al.[1] As was seen for nitrogen isotopes, the oxygen isotope ratio of product N_2O, shown here, reaches a constant value upon complete conversion of nitrate to N_2O, typically after only 30 min (Figure 2A). The $^{18}O/^{16}O$ values are then stable for incubations of at least 16 h (Figure 2B), which is the time scale for preparation and analysis of a batch of samples; other analyses (not shown) indicate no change in precision for at least 72 h. The change in $\delta^{18}O$ of N_2O between the first measurement (after 2 min) and the final measurement is small (3‰) compared to the change in $\delta^{15}N$ (15‰) over the same time course. This small variation in $\delta^{18}O$ is important for the precision of oxygen isotope results for *P. aureofaciens*, as slightly incomplete conversion would not impart a large isotopic error.

Underlying this small change in the $^{18}O/^{16}O$ ratio of the product over the course of conversion is a much larger kinetic isotope effect associated with the removal of oxygen atoms from nitrate, which causes the N_2O to be enriched in ^{18}O compared to the initial nitrate. The $\delta^{18}O$ value of N_2O produced from N3 is typically +20‰ relative to our reference N_2O tank. The $\delta^{18}O$ value of our N_2O reference tank has not been determined precisely, but analyses of atmospheric N_2O suggest that the tank has a $\delta^{18}O$ value close to other industrial N_2O tanks, which range between 36 and 43‰ versus VSMOW.[26] This would mean that the $\delta^{18}O$ of the N_2O produced from N3 is approximately +60‰ versus VSMOW and about +40‰ relative to the starting nitrate. This observation can be explained by preferential loss of ^{16}O during the enzymatic reduction of nitrate, which leads to enrichment of ^{18}O in the product, N_2O. Although the isotopic fractionation between nitrate and the product N_2O is large (40‰), it is consistent among analyses from a single culture batch, as seen in the achievement of a constant $\delta^{18}O$ value among replicate analyses of N3 that have undergone complete conversion (Figure 2). Thus, while fractionation affects the measured $\delta^{18}O$ of individual samples (eq 2), this effect is removed by normalizing to analyses of a nitrate reference with a known $\delta^{18}O$ value.

Oxygen Exchange with Water. Because correcting properly for oxygen exchange processes is important to the accuracy of $\delta^{18}O$ analyses, we carried out experiments to further characterize the amount of exchange catalyzed by *P. aureofaciens*, *P. chlororaphis*, and *C. nephridii* (another N_2O-producing denitrifier) under the conditions of this application. Exchange was quantified by measuring incorporation of oxygen from ^{18}O-labeled water into

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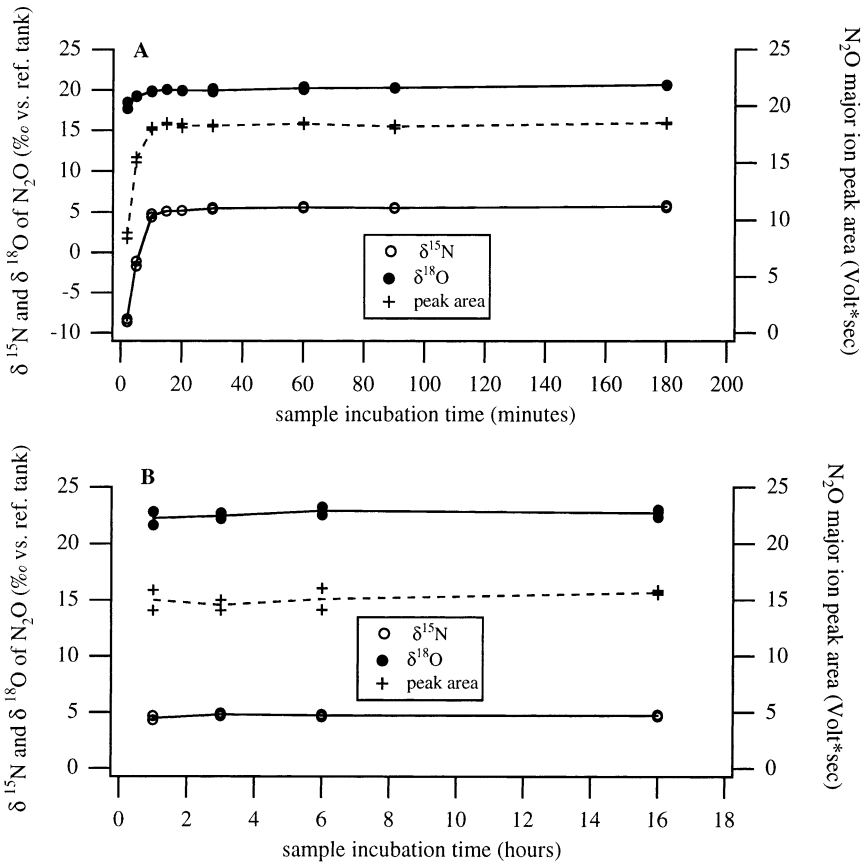

Figure 2. Time course of the yield (peak area of the $m/z = 44$ cup), $\delta^{15}N$, and $\delta^{18}O$ of N_2O produced from IAEA-NO-3 by *P. aureofaciens*. Each point represents a separate vial to which 1 mL of 20 μM IAEA-NO-3 had been added and allowed to react for periods of time ranging from 10 to 180 min (A) and extended to 16 h (B). N_2O yield and $\delta^{15}N$ data reprinted from Sigman et al.[1]

N_2O during conversion from nitrate. N3 was diluted to 20 μM in waters with $\delta^{18}O$ values ranging from −6.82 to +325‰. These N3 solutions were added (0.75 or 1.0 mL) to prepared vials of each strain, which completely converted the nitrate to N_2O.

From eq 2, assuming m, b, s, ϵ, and $\delta^{18}O_b$ are constant for a batch of samples, one sees that the $\delta^{18}O_m$ of a single standard (constant $\delta^{18}O_s$) will vary with $\delta^{18}O_{H_2O}$ and exchange (x). The exchange is given by the ratio $(m/s)(\delta^{18}O_{m,1} - \delta^{18}O_{m,2})/(\delta^{18}O_{H_2O,1} - \delta^{18}O_{H_2O,2})$, or the slope of the linear regression of $\delta^{18}O_m$ versus $\delta^{18}O_{H_2O}$ when corrected for the blank, assuming that the blank originates from previously produced N_2O (Figure 3). Full exchange would yield a line with slope of 1 on this plot (or slightly less than 1, due to the blank) whereas a reaction with no exchange would show no change in $\delta^{18}O_m$ with increasing $\delta^{18}O_{H_2O}$ (zero slope). Our experiments show that incorporation of oxygen from H_2O into N_2O during denitrification by *P. aureofaciens* is less than 10% and frequently lower than 3%. *C. nephridii* has an intermediate level of exchange (29.7%), although it also has the copper-type nitrite reductase.[21] *P. chlororaphis* has a much higher level of exchange (61.0−78.1%), which tends to increase with culture age (data not shown). Thus, under these conditions, *P. aureofaciens* consistently had the lowest exchange, consistent with the findings of Ye et al.,[19] and is the organism that we use routinely for nitrate $\delta^{18}O$ analysis.

The strong linear regressions seen in Figure 3 ($r^2 > 0.98$ for series with four different $\delta^{18}O_{H_2O}$ values) and the isotopic reproducibility of enriched samples are indications that exchange is constant for a given batch of analyses, even though this exchange does vary among culture batches. While the exchange for *P. aureofaciens* is low, for the most accurate $\delta^{18}O$ measurements, it is important to measure the exchange for each set of samples (i.e., for a given culture batch). The observed range in exchange values for *P. aureofaciens* in the experiments shown in Figure 3 is 2.4−8.7%; if we assume an average value of 5%, our calculated $\delta^{18}O_s$ for a sample with a true $\delta^{18}O_s$ of +20‰ relative to N3 would be in error by ±0.6 ‰ on days when the actual exchange was 2 or 8%.

Blanks. As discussed previously,[1] N_2O that remains closely associated with the bacteria throughout the sample vial preparation but is subsequently extracted during sample analysis gives rise to a methodological blank. In method testing, the amount of blank decreased as the preparative purging time of the sample vials was extended from 20 min to 2 h.[1] A 3-h purging time is now recommended for consistently low blanks (less than 0.5 nmol). Because this blank is associated with the bacteria themselves (or the culture medium), it may vary between different batches of bacteria but does not vary significantly within samples prepared from a single batch of bacteria. Therefore, when the culture blank size is measured with each batch, it can be applied as a constant correction to all samples run on that day.

Reproducibility. The within- and among-day reproducibility of $\delta^{18}O$ measurements was investigated using *P. aureofaciens*. To address within-day reproducibility, the standard deviation of $\delta^{18}O_m$ values (vs tank) for replicate N3 analyses was calculated for each

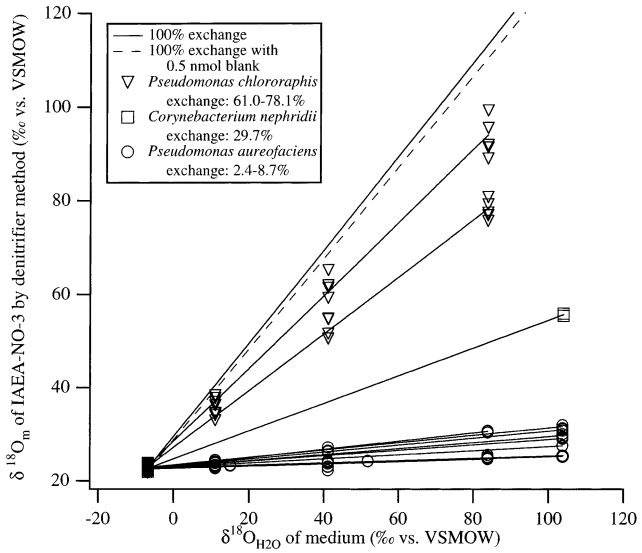

Figure 3. Incorporation of ^{18}O into N_2O from ^{18}O-labeled water during denitrification. IAEA-NO-3 was diluted to 20 μM in water of $\delta^{18}O$ values ranging from −6.8 to +325‰ (measured in the laboratory of D. Schrag). These IAEA-NO-3 solutions (0.75 or 1.0 mL) were injected into prepared vials of *P. aureofaciens*, *P. chlororaphis*, and *C. nephridii* and converted completely to N_2O. The $\delta^{18}O_m$ values of N_2O produced from IAEA-NO-3 are plotted against the $\delta^{18}O_{H_2O}$ of the medium used for conversion. Individual analyses (open symbols) are plotted for each series. The fraction of oxygen replacement due to nitrogen oxide/water exchange was calculated for each strain from the slopes of the regression lines shown here. Twelve separate experiments for *P. aureofaciens*, two experiments for *P. chlororaphis*, and one experiment for *C. nephridii* are shown. Also shown are the trends calculated for complete oxygen exchange during the conversion (upper solid line) and the effect of an uncorrected 0.5-nmol blank on that calculation (dashed line).

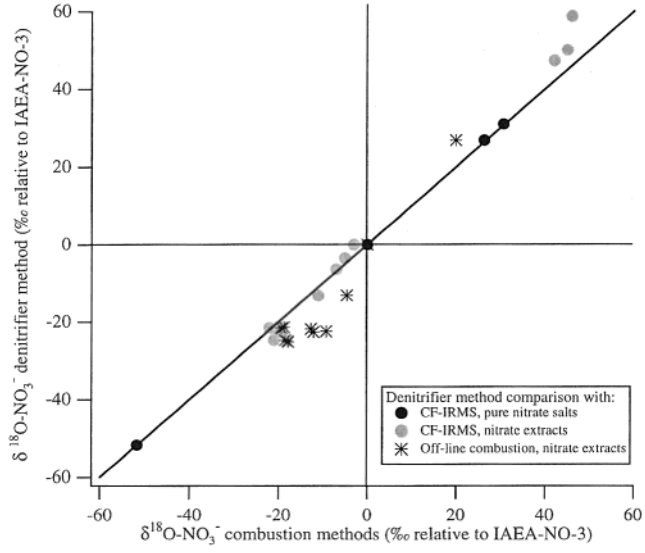

Figure 4. Direct comparison of the denitrifier method to combustion-based methods for $\delta^{18}O$ analyses. The averages of replicate $\delta^{18}O$ measurements for each sample are shown as differences from the average of $\delta^{18}O$ measurements for IAEA-NO-3 measured on the same day. The analyses from the denitrifier method are plotted vs early off-line combustion-based measurements (asterisks) and vs later measurements by CF-IRMS (circles). Among the samples that compare the denitrifier method and CF-IRMS, black circles indicate salt samples that could be directly analyzed by CF-IRMS and gray circles indicate precipitation and groundwater samples that required purification previous to analysis by CF-IRMS. Complete agreement between denitrifier and combustion analyses would follow the indicated 1:1 line.

of 10 days, each with four or more analyses of N3. The standard deviation on different days ranged from 0.13 to 0.59‰, with an average of 0.34‰. This relatively low standard deviation is important for the precision of the $\delta^{18}O$ measurement using the denitrifier method, and it is a further demonstration of the consistency of fractionation, exchange, and blank among samples from a single batch of bacteria.

To evaluate the reproducibility of $\delta^{18}O$ measurements among days (and different batches of bacteria), we analyzed aliquots of a second nitrate solution (RSIL-35a). Reproducibility among days requires accurate assessment and correction for blanks, exchange, and fractionation, as described in the Corrections for Oxygen Isotopic Fractionation, Exchange, and Blank section (above). The $\delta^{18}O_m$ for RSIL-35a was measured for 10 days of analysis and corrected for blank and exchange according to eq 4, using $\delta^{18}O_{s,ref}$ of 22.7‰ versus VSMOW for N3. The $\delta^{18}O_s$ of RSIL-35a ranged from 52.9 to 54.25‰ versus VSMOW, with an average value of 53.84 ± 0.42‰ (1σ SD). For these samples, the measured variation in exchange (0.2−5.5%) equates to corrections of 0.07−1.66‰, while the variations in blank (0.22−0.47 nmol) equates to corrections of 0.45−0.91‰. The uncorrected $\delta^{18}O_m$ values for RSIL-35a ranged from 48.68 to 51.44‰, showing a greater range and standard deviation (0.95‰) compared to the corrected $\delta^{18}O_s$ values.

Comparison with Combustion Methods. The $\delta^{18}O$ values of several nitrate samples were measured both by the denitrifier method and by published methods for off-line combustion[14] or on-line combustion with continuous-flow isotope ratio mass spectrometry (CF-IRMS).[17] Aliquots of KNO_3 and $NaNO_3$ salts and several groundwater and precipitation samples were analyzed. For analysis by the denitrifier method, the samples were diluted to 20 μM solutions and analyzed in 10-nmol aliquots. Analyses by combustion methods were conducted using dried nitrate salts (approximately 3 μmol of N for CF-IRMS or 50−100 μmol of N for off-line combustion). For groundwater and precipitation samples, the denitrifier method was applied to filtered, but otherwise untreated samples, whereas the combustion-based methods required additional purification of the nitrate using anion exchange and precipitation of the nitrate salts. The results for the different methods were compared by expressing all $\delta^{18}O$ values relative to that of N3, which was analyzed by each of the methods.

Results from the denitrifier method and CF-IRMS agree well for many groundwater, precipitation, and salt samples (Figure 4). In particular, the agreement is within ±0.5‰ for three nitrate salts with widely varying $\delta^{18}O$ values, RSIL-N34a (−52‰ vs N3), RSIL-N35a (+31‰ vs N3), and Chile 375 (+26‰ vs N3).

For some groundwater and precipitation samples, there were minor discrepancies between the denitrifier and CF-IRMS analyses, particularly for samples with high $\delta^{18}O$ values. The denitrifier method generally yielded slightly larger $\delta^{18}O$ differences between these samples than the CF-IRMS method. Both exchange of oxygen atoms with other oxides and the introduction of blank (analyte not originating from the NO_3^- sample) will lead to loss of primary nitrate isotope signal and underestimation of $^{18}O/^{16}O$ differences among samples. Given that the blanks are well-

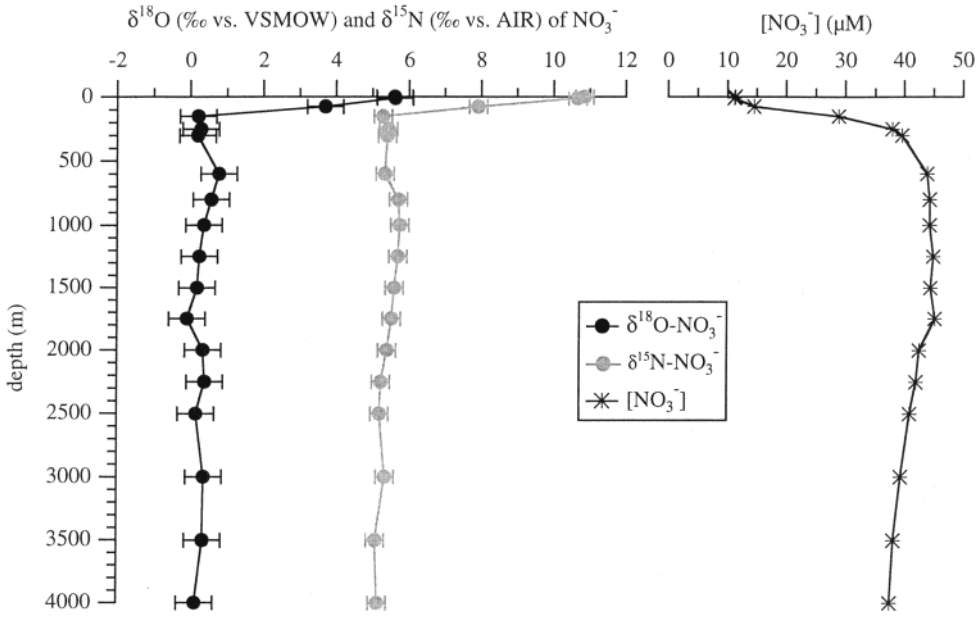

Figure 5. Nitrate $\delta^{15}N$ and $\delta^{18}O$ (left) and nitrate concentration (right) for a depth profile collected in July 1999 at station P (50° N, 145° W) in the Gulf of Alaska, subarctic North Pacific. Nitrate $\delta^{15}N$ and $\delta^{18}O$ values are plotted as averages of multiple analyses ($n = 2-3$). Error bars denote typical standard deviations (1σ) for isotopic analysis, 0.2‰ for $\delta^{15}N$ and 0.5‰ for $\delta^{18}O$. $\delta^{18}O$ values are reported vs VSMOW by assuming a value of 22.7‰ for IAEA-NO-3. Blank size was not measured with roughly a third of these samples but was assumed to be 0.5 nmol for the purpose of $\delta^{18}O$ corrections. For the range of blank sizes that we typically observe (0.2−0.7 nmol), this imposes an added uncertainty of ±0.3‰.

characterized, we suspect that the slight discrepancy is due to exchange associated with some part of the combustion-based protocol. The agreement for solid nitrate salts suggests that this error may not be associated with the CF-IRMS combustion itself but with the nitrate purification steps that are required when groundwater and precipitation samples are to be analyzed by CF-IRMS.

Several samples analyzed by the off-line combustion-based method[14] yielded relatively poor agreement with the denitrifier method (and with the CF-IRMS results), with the off-line combustion method consistently yielding lower $\delta^{18}O$ differences between samples and N3 (Figure 4). Again, our suspicion is that the discrepancy originates in the method that yields smaller $\delta^{18}O$ differences among samples, in the case, the off-line combustion method. Since blank size has been characterized for the off-line combustion method, our presumption is that these larger discrepancies are the result of substantial oxygen exchange with an unknown substrate. While sample preparation for combustion may control some fraction of the error (as discussed above), the large differences in analyses between the two combustion methods for samples that underwent the same sample preparation suggests that the main issue with the off-line combustion method is oxygen exchange during the combustion step. We suspected that the contaminating pool of oxygen atoms is associated with the quartz glass tubing used in the off-line combustion; an intercomparison of combustion methods appears to confirm this suspicion (Revesz, K.; Böhlke, J. K. *Anal. Chem.*, in press). This source of contamination is not present in the CF-IRMS method, which uses a glass-free carbon reactor. We cannot evaluate the generality of this problem among different combustion-based approaches; however, our intercomparison efforts to this point indicate that exchange is an important parameter to be characterized in methods for nitrate oxygen isotope analysis and that the denitrifier method has better accuracy than at least some combustion methods.

Application to Seawater Samples. To illustrate the potential of the denitrifier method, we report nitrogen and oxygen isotope ratios for a depth profile of seawater nitrate samples from the Gulf of Alaska in the eastern subarctic Pacific (Figure 5). To our knowledge, these are the first published data on the $\delta^{18}O$ of oceanic nitrate. Two major observations arise from these data.

The first observation is that the $\delta^{18}O$ of nitrate in the deep ocean is close to 0‰ versus VSMOW. Biochemical studies have derived mechanisms for ammonium oxidation to nitrite (NO_2^-) in which one oxygen atom is donated from O_2 and the other from H_2O.[27] Nitrite oxidation to nitrate (NO_3^-) involves the donation of oxygen only from water.[28] On this basis, the traditional interpretation has been that one-third of the oxygen atoms in nitrate should originate from O_2.[10,11,12,29] Since O_2 in the ocean interior has $\delta^{18}O$ values between 23.8 and 35.5‰,[30] it may thus seem surprising that the $\delta^{18}O$ of oceanic nitrate is close to seawater $\delta^{18}O$. However, the biochemical studies that provided the evidence for a 1:2 O_2/water oxygen source ratio also demonstrated a strong nitrifier-catalyzed nitrite−water exchange.[27] We have observed similar nitrite−water oxygen isotope exchange in experiments with ammonium oxidizers (K. L. Casciotti, unpublished). Based on these observations, less than one in six (and perhaps none) of the oxygen atoms in marine (and freshwater) nitrate comes from O_2. It is also possible that catalysis of exchange with water occurs during the oxidation of nitrite to nitrate.[31] Thus,

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despite previously published interpretations, we would argue that one should, in fact, expect the $\delta^{18}O$ of newly produced nitrate to be close to the $\delta^{18}O$ of the ambient water. Even if one in six oxygen atoms in nitrate is from O_2, given the relatively small range in $\delta^{18}O$ of dissolved O_2[30] and the fact that the in situ nitrate pool in any water parcel has been generated over a range of O_2 concentrations and $\delta^{18}O$ values, the net effect of O_2 isotopic variation on nitrate $\delta^{18}O$ variation should be minor in most marine environments. Thus, we should expect that nitrate that is produced in the deep sea has a $\delta^{18}O$ that is near that of VSMOW and that varies little due to nitrification—both expectations are supported by the data presented in Figure 5.

It must be admitted that we cannot currently have great confidence in whether the $\delta^{18}O$ of deep ocean nitrate is slightly greater or slightly less than VSMOW. This uncertainty is due largely to the fact that our isotopic reference, N3 (as with all industrial nitrate salts), has a high $\delta^{18}O$ and the errors in our corrections for blank and water exchange are amplified for low-$\delta^{18}O$ samples. Furthermore, the $\delta^{18}O$ values reported here may be as much as 3‰ too low if the correct $\delta^{18}O$ value for N3 is higher than 22.7‰ versus VSMOW, as is seen in some studies.[17] This combined uncertainty underscores the great need for the development of a set of nitrate oxygen isotope reference materials.

The second major observation from the subarctic Pacific depth profile is that, as is well established for the nitrogen isotope ratios of nitrate, there is an oxygen isotopic fractionation associated with the consumption of nitrate by phytoplankton in ocean surface waters that leaves the residual nitrate enriched in ^{18}O. Comparison of the increases in nitrate $\delta^{18}O$ and $\delta^{15}N$ from the subsurface into the surface layer indicates a ratio of isotope discrimination (or a ratio of isotope effects, $^{18}\epsilon/^{15}\epsilon$) that is close to unity (1.03 ± 0.05, 1σ SD). The oxygen isotope effect promises to add a critical constraint in efforts to understand the specific biochemical origin of the isotope discrimination that occurs in nitrate uptake, in that different steps in nitrate assimilation are likely to alter the $^{15}N/^{14}N$ and $^{18}O/^{16}O$ ratios in different ways. Moreover, if this $^{18}\epsilon/^{16}\epsilon$ ratio proves to be predictable in the ocean, then it will represent a powerful tool to study ocean circulation and nitrogen cycle processes. As would be expected based on the freshwater literature,[32] denitrification in the ocean also causes both nitrogen and oxygen isotope fractionation.[33] Taking these results together, it appears that the oxygen isotopic variations that we will observe in the ocean will be due primarily to the removal of nitrate, not its production.

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CONCLUSION

The denitrifier method is the first method for $^{18}O/^{16}O$ analysis of nitrate in seawater. Moreover, it has many benefits over other currently available methods for oxygen isotopic analysis of nitrate in freshwaters. The denitrifier method achieves a much higher level of sensitivity than published methods, providing similar precision for $\delta^{18}O$ with 2−3 orders of magnitude less nitrate required per analysis. This reduction in sample size allows for analysis of samples with low nitrate concentrations (down to 1 μM) and limited volumes (10 mL for 1 μM NO_3^-). Blank size, oxygen isotope fractionation, and incorporation of non-nitrate oxygen atoms into the N_2O analyte are adequately reproducible for a given batch of samples to be easily corrected using standards run in each batch of samples. Comparison of the denitrifier method with previously published combustion-based methods indicates that oxygen atom exchange is potentially important in off-line combustion-based methods and requires further characterization.

One of the main obstacles we have confronted in this development effort is the paucity of oxygen isotope reference materials for nitrate. At least two well-known reference materials are required to correct nitrate $\delta^{18}O$ measurements for the dual influence of oxygen exchange and blank size, which we have shown to be significant both in our method and other available (i.e., freshwater) methods.

ACKNOWLEDGMENT

We gratefully acknowledge D. Schrag for $\delta^{18}O$ analyses of H_2O. We thank G. Cane, R. Ho, A. Trompeo, K. Revesz, S. Mroczkowski, and J. Hannon for technical assistance and B. B. Ward and J. M. Tiedje for helpful discussion. T. Coplen and M. Voytek provided constructive reviews of the manuscript. We also thank P. Tortell for collection of samples from the subarctic Pacific and F. Whitney for nitrate concentration data for these samples. This work was supported by NSF grants OCE99-81479 and OCE00-81686 to D.M.S., as well as by British Petroleum and Ford Motor Co., through the Princeton Carbon Mitigation Initiative. Support for K.L.C. was provided by the NSF grant OCE96-17690 to B.B.W. and the Center for Environmental Bioinorganic Chemistry. Funding for J.K.B. was provided by the National Research Program of the USGS Water Resources Division. M.G.H. acknowledges support from a NSF Graduate Research Fellowship.

Received for review February 20, 2002. Accepted July 31, 2002.

AC020113W