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 (18O/16O) of nitrate (NO3–) 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 (15N/14N) analysis of nitrate.¹ Here, we address the additional issues associated with 18O/16O analysis of nitrate by this approach, which include (1) the oxygen isotopic difference between the nitrate sample and the N2O 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 N2O. Experiments with 18O-labeled water indicate that water exchange contributes less than 10%, and frequently less than 3%, of the oxygen atoms in the N2O product for Pseudomonas aerofaciens. In addition, both oxygen isotopic 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 18O/16O ratios of samples for both effects. This is the first method tested for 18O/16O 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 NO3–), lack of interference by other solutes, and ease of sample preparation.

Nitrate is the dominant form of bioavailable nitrogen in the ocean. The 15N/14N ratio in nitrate is an important integrative tool for understanding internal cycling (assimilation, remineralization, nitrification) and transport of nitrate as well as the balance of inputs and losses of nitrogen in the ocean (nitrogen fixation and denitrification).²⁻⁴ 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.⁵⁻¹² This same complementarity will likely apply in the ocean, but the lack of a method for 18O/16O analysis of nitrate in seawater has precluded the use of nitrate oxygen isotopes as a constraint on oceanic nitrogen transformations.

Published methods for 18O/16O analysis of nitrate from freshwaters are based on collection of nitrate on anion exchange columns and high-temperature conversion of nitrate to N2 and CO2 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 SO4²⁻ concentrations and typically low NO3⁻ 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 15N/14N analysis of nitrate, described by Sigman et al.,¹ is based on bacterial conversion of nitrate to N2O by Pseudomonas aureofaciens.¹³ Because 18O is a light oxygen isotope that is preferentially lost from nitrate during nitrification and transport of nitrate²,³ as well as the balance of inputs and losses of nitrogen in the ocean (nitrogen fixation and denitrification),¹² the oxygen isotopic method for nitrate analysis can be used to infer the nitrogen isotopic composition of nitrate from seawater samples.

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N₂O and, therefore, can also offer oxygen isotope information. While the conversion of nitrate to N₂O 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 N₂O analyte:

\[ 2\text{NO}_3^- \rightarrow 2\text{NO}_2^- \rightarrow 2\text{NO} \rightarrow \text{N}_2\text{O} \]

If there is preferential loss of $^{16}\text{O}$ in these reactions, there will be a difference in $^{18}\text{O}/^{16}\text{O}$ between nitrate and the product N₂O, even with complete conversion of nitrate to N₂O. This oxygen isotopic fractionation must be adequately reproducible for a given batch of analyses to properly correct for this effect.

The second concern in $^{18}\text{O}/^{16}\text{O}$ 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 N₂O analyte. Enzymatic catalysis of the exchange of water oxygen with nitrite ($\text{NO}_2^-$) 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 exchange¹⁹ ($39-76\%$), 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 N₂O (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 $^{18}\text{O}/^{16}\text{O}$ 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. chlorophilus), P. chlororaphis (provided by J. M. Tiedje), and Corynebacterium nephridii (ATCC 11425), are maintained on Tryptic Soy Agar (Difco), amended with 10 mM KNO₃. Starter cultures (5-mL ATCC 11425), are maintained on Tryptic Soy Agar.

While the conversion of nitrate to N₂O represents a mass balance 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 N₂O analyte:

\[ 2\text{NO}_3^- \rightarrow 2\text{NO}_2^- \rightarrow 2\text{NO} \rightarrow \text{N}_2\text{O} \]

**¹⁸O/¹⁶O 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 N₂. 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 N₂O before the addition of 0.1 mL of 10 N NaOH to stop bacterial activity and scavenge CO₂. A constant sample size (e.g., 20 nmol) is targeted for each batch of samples to simplify the correction of $\delta^{18}\text{O}$ values (below). In early method development and application, the N₂O analyte was extracted from sample vials offline and analyzed using a Finnigan PreCon upstream of a Finnigan MAT 252 IRM S.³ Subsequently, we adapted a Finnigan GasBench II system for on-line automated extraction and analysis of N₂O (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 CombiPalm 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 N₂O 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-050-725S) and a chemical trap (layered magnesium perchlorate and ascarite) to remove water and CO₂ 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 N₂ (LN₂) to condense N₂O (as well as any remaining H₂O and CO₂), while the uncondensed gases (He, N₂) 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 i.d. glass capillary, which is not immersed in LN₂ 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 LN₂, and after a 60-s delay to allow T2 to reach LN₂ temperatures, T1 is raised from LN₂. After another 340 s, when transfer of sample gas from T1 to T2 is complete, T2 is raised from LN₂. 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 N₂O is separated from residual CO₂ 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.³ During inject mode, the chemical trap,
first Nafion water trap, and autosampler outflow needle are back-flushed with He (Figure 1).

A Finnigan DELTAplus isotope ratio mass spectrometer is used in continuous-flow mode to measure the $^{\text{18}}$O/$^{\text{16}}$O)45/44 and 46/44 ratios of each sample. These ratios are measured against pulses of reference N2O 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 N2O 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 KNO3 reference material with an assigned $\delta^{15}$N of 4.7½ versus atmospheric N2 24,25 and reported $\delta^{18}$O of 22.7½-25.3½ versus VSMOW 14,16,17 ($\delta^{18}$O) sample (VSMOW) = [($^{18}$O/$^{16}$O) sample (VSMOW) - 1] x 1000. For the purposes of this study, we adopt a value of 22.7½.

**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 N2O depends on isotopic fractionation, exchange, and blank according to the following mass and isotope balance equations:

$$m = s + b$$  \hspace{1cm} (1)

$$\delta^{18}O_m = (\delta^{18}O_s + \epsilon)(1 - x) + \delta^{18}O_{H_2O}sx + \delta^{18}O_{b}b$$  \hspace{1cm} (2)

where m is the total amount of measured N2O 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 N2O 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. $\epsilon$ is the net isotopic fractionation caused by the removal of oxygen atoms in the reduction of nitrate to N2O. The use of $\delta$ 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 1½% 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, isotopic fractionation, and blank size are constant among samples prepared with

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**Figure 1.** Automated N2O extraction and $\delta^{18}$O analysis system. In load mode (A), the N2O 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 N2O 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).
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:

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

(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 \(\delta^{18}O\) 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 \(\delta^{18}O\) of each sample for the cumulative effect of blank, exchange, and fractionation using the following equation:

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

(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 \(\delta^{18}O_{s,N} \) and \(\delta^{18}O_{m,N}\) for \(N_3\) in normal and \(^{18}O\)-enriched water (\(\delta^{18}O_{s,N} = 325\%\)), 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, \(N_3\) (\(\delta^{18}O_{s,ref}\) 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 \(N_3\). The uncertainty is proportionally lower for samples that are more similar in \(\delta^{18}O\) to \(N_3\).

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 acidic (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 nitric 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. 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 \(\delta^{18}N\) (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 \(N_3\) 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. This would mean that the \(\delta^{18}O\) of the \(N_2O\) produced from \(N_3\) is typically +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 \(N_3\) 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.

N$_2$O during conversion from nitrate. N$_3$ was diluted to 20 $\mu$M in waters with $\delta^{18}$O values ranging from $-6.82$ to $+325\%$. These N$_3$ solutions were added (0.75 or 1.0 mL) to prepared vials of each strain, which completely converted the nitrate to N$_2$O.

From eq 2, assuming $m$, $b$, $s$, and $\delta^{18}$O$_{H_2O}$ are constant for a batch of samples, one sees that the $\delta^{18}$O$_m$ of a single standard (constant $\delta^{18}$O$_{H_2O}$) 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$_2$O (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$_2$O into N$_2$O 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. 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., 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% for our calculated $\delta^{18}$O$_m$ for a sample with a true $\delta^{18}$O$_m$ of $+20\%$ relative to N$_3$ would be in error by $\pm0.6\%$ on days when the actual exchange was 2 or 8%.

**Blanks.** As discussed previously, N$_2$O 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. 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 N$_3$ analyses was calculated for each
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 \( \delta^{18}O_{\text{m}} \) for RSIL-35a was measured for 10 days of analysis and corrected for blank and exchange according to eq 4, using \( \delta^{18}O_{\text{ref}} \) of 22.7%versus VSMOW for N3. The \( \delta^{18}O_{\text{m}} \) 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 \( \delta^{18}O_{\text{m}} \) values for RSIL-35a ranged from 48.68 to 51.44‰ showing a greater range and standard deviation (0.95‰) compared to the corrected \( \delta^{18}O_{\text{m}} \) 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-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.
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 the slight discrepancy is due to exchange associated with some part of the combustion-based protocol. The agreement for solid nitrate salts suggests 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 exchange associated with some part of the combustion-based 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 exchange associated with some part of the combustion-based protocol. The agreement for solid nitrate salts suggests that this exchange associated with some part of the combustion-based protocol. The agreement for solid nitrate salts suggests that this exchange associated with some part of the combustion-based protocol. The agreement for solid nitrate salts suggests that this exchange associated with some part of the combustion-based protocol. The agreement for solid nitrate salts suggests that this exchange associated with some part of the combustion-based protocol. The agreement for solid nitrate salts suggests that this exchange associated with some part of the combustion-based protocol. The agreement for solid nitrate salts suggests that this exchange associated with some part of the combustion-based protocol. The agreement for solid nitrate salts suggests that this exchange associated with some part of the combustion-based protocol. The agreement for solid nitrate salts suggests that this exchange associated with some part of the combustion-based protocol. The agreement for solid nitrate salts suggests that this exchange associated with some part of the combustion-based protocol.

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 ($\text{NO}_2^-$) in which one oxygen atom is donated from O$_2$ and the other from H$_2$O. Nitrite oxidation to nitrate ($\text{NO}_3^-$) involves the donation of oxygen only from water. On this basis, the traditional interpretation has been that one-third of the oxygen atoms in nitrate should originate from O$_2$. Since O$_2$ in the ocean interior has $\delta^{18}O$ values between 23.8 and 35.5‰, 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 nitritifier-catalyzed nitrite—water exchange. 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. Thus,

$$
\delta^{18}O_{\text{NO}_3^-} = \delta^{18}O_{\text{NO}_2^-} + \delta^{18}O_{\text{H}_2\text{O}}
$$

Figure 5. Nitrates $\delta^{18}N$ 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. Nitrates $\delta^{18}N$ and $\delta^{18}O$ values are plotted as averages of multiple analyses ($n = 2$) Error bars denote typical standard deviations (1σ) for isotopic analysis. 0.2‰ for $\delta^{18}N$ 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‰.

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, N\(_3\) (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 N\(_3\) is higher than 22.7\% versus VSMOW, as is seen in some studies.\(^{31}\) 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, \( \delta_{15}N / \delta_{18}O \)) 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 \( \delta_{15}N / \delta_{18}O \) 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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