A Bacterial Method for the Nitrogen Isotopic Analysis of Nitrate in Seawater and Freshwater

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We report a new method for measurement of the isotopic composition of nitrate (NO$_3^-$) at the natural-abundance level in both seawater and freshwater. The method is based on the isotopic analysis of nitrous oxide (N$_2$O) generated from nitrate by denitrifying bacteria that lack N$_2$O-reductase activity. The isotopic composition of both nitrogen and oxygen from nitrate are accessible in this way. In this first of two companion manuscripts, we describe the basic protocol and results for the nitrogen isotopes. The precision of the method is better than 0.2‰ (1 SD) at concentrations of nitrate down to 1 μM, and the nitrogen isotopic differences among various standards and samples are accurately reproduced. For samples with 1 μM nitrate or more, the blank of the method is less than 10% of the signal size, and various approaches may reduce it further.

Nitrate (NO$_3^-$) is the predominant form of bioavailable (or “fixed”) nitrogen in the ocean, and the natural isotopic variations of this species provide an important tool for studying the nitrogen cycle. Depending on the environment, the $^{15}$N/$^{14}$N ratio of seawater nitrate can provide information on virtually all of the major transformations of nitrogen that occur in the ocean, including dinitrogen fixation, uptake of fixed nitrogen by phytoplankton, nitrification, and denitrification. The $^{18}$O/$^{16}$O ratio of nitrate has been studied in freshwater and terrestrial systems and has been shown to provide an additional important constraint on natural processes. Generally, both the nitrogen and oxygen isotopic compositions of nitrate have many potential applications in oceanography, hydrology, and atmospheric chemistry; however, natural-abundance isotopic studies of nitrate have been restricted by analytical limitations, especially in marine systems.

Methods to measure the nitrogen isotopic composition of nitrate in natural waters typically involve the reduction of nitrate to ammonia, followed by extraction of ammonia using diffusion or distillation, reaction to N$_2$ gas, and isotopic analysis of the N$_2$ (ref 2 and references therein). Methods also exist for the coupled nitrogen and oxygen isotopic analysis of nitrate in freshwater that are based on purification of the nitrate salt and the direct, high-temperature conversion of nitrate to N$_2$ and CO$_2$. However, weaknesses in the available methods have made some nitrate isotope investigations difficult and have precluded others. The published N$_2$-based methods for nitrogen isotopic analysis normally require micromoles of nitrate-N, which is prohibitive when only milliliters of water are available. The methods available for the nitrogen isotopic analysis of seawater nitrate require nitrate concentrations of 2–3 μM or higher, largely because of the limited efficiency with which ammonia is extracted by distillation and diffusion. In addition, these methods have a significant reagent blank and a blank associated with dissolved organic nitrogen that varies with sample type and can be large. Finally, these methods are typically labor- or time-intensive. With respect to oxygen isotopic analysis, the weaknesses in the available methods are even more restrictive. In particular, there is no published method for the oxygen isotopic analysis of nitrate in seawater.

We describe here a bacterial method for measuring the isotopic composition of seawater nitrate at the natural-abundance level. The method is based on the analysis of nitrous oxide gas (N$_2$O) that is produced quantitatively from nitrate by denitrifying bacteria. The classical denitrification pathway consists of the stepwise reduction of nitrate (NO$_3^-$) to nitrite (NO$_2^-$), nitric oxide (NO), nitrous oxide (N$_2$O), and dinitrogen (N$_2$):

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

Each of these steps is carried out by a dedicated enzyme encoded by a distinct gene. There is a rich literature on natural and genetically modified bacterial strains that lack discrete components of the denitrification pathway. The method described below takes advantage of naturally occurring denitrifiers that lack an active N$_2$O reductase, the enzyme that reduces N$_2$O to N$_2$.

Previous workers developed this and related approaches for the measurement of nitrate concentration and $^{15}$N tracer incorpora-

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Our work focuses on achieving the levels of isotopic precision and accuracy required for natural-abundance studies at nitrate concentrations that are broadly relevant to oceanographic and other environmental questions. This method has many advantages relative to our previous method of choice:2 a 100-fold reduction in the sample size requirement, a reduction in the proportional blank size for samples with lower nitrate concentrations, a reduction in the time requirement of the analysis, the reproducible isotopic analysis of samples down to 1 µM nitrate, and the ability to analyze the oxygen isotope composition of seawater nitrate. Additional improvements should be obtainable with continued development of the method.

For both the nitrogen and oxygen isotopes, isotopic fractionation is observed during the bacterial reduction of nitrate to nitrous oxide. For the nitrogen isotopes, if the conversion is complete and there are no additional nitrogen pools being converted to N₂O, mass balance requires that the δ¹⁵N of the product N₂O is identical to that of the initial nitrate. Although the conversion of nitrate to N₂O in this system represents a mass balance reaction with respect to nitrogen, this is not the case for oxygen. Only one of six oxygen atoms present in the initial nitrate pool is preserved in the product N₂O. If the isotopic fractionations associated with this redistribution of oxygen atoms vary, then the δ¹⁸O value of the N₂O product will vary even if that of the nitrate source does not. Another concern involves the exchange of oxygen atoms between the various nitrogen oxide intermediates and water during the conversion of nitrate to N₂O, which would dilute or remove the oxygen isotopic signal of the nitrate sample. These additional concerns for the oxygen isotopic analysis of nitrate by the denitrifier method have prompted additional testing and intercalibration to demonstrate the accuracy of the method. For reasons of focus and brevity, we limit ourselves here to a description of the basic protocol and results relevant to the nitrogen isotopic analysis of nitrate, leaving a full description of the oxygen isotopic analysis of nitrate by the denitrifier method for a separate manuscript (K. L. Casciotti et al., in preparation).

In the Experimental Section, we provide a complete description of the protocol for isotopic analysis of nitrate nitrogen via the denitrifier method, as well as background and additional information relevant to application of the method. In the Results and Discussion Section, we report isotopic results that document the precision and accuracy of the method. In addition, we report nitrogen isotopic compositions for nitrate from the pore waters of marine sediments as an example of the new sample types that can be analyzed with the denitrifier method.

**EXPERIMENTAL SECTION**

Denitrifier Strains. Both Pseudomonas chlororaphis (ATCC # 43928, deposited by J. M. Tiedje) and Pseudomonas aureofaciens (ATCC #13985, recently reclassified as a strain of P. chlororaphis) lack nitrous oxide reductase activity.8,9,11 Both strains work well for nitrogen isotopic measurements and have similar characteristics with regard to reaction times and blanks. As will be described elsewhere, conversion by P. aureofaciens also allows for determination of the oxygen isotopic composition of nitrate. While P. chlororaphis cannot be used for oxygen isotopic analysis, it has proven to be more robust in culture.

**Preparation of Denitrifier Cultures.** Tryptic soy broth (Difco Laboratories) is amended with 10 mM potassium nitrate, 1 mM ammonium sulfate, and 1 mL/L of an antifoaming agent (Dow-Corning formula B). Medium is dispensed by 400-mL aliquots into 500-mL media bottles and autoclaved. Once autoclaved, the medium can be stored for at least a month. The appropriate strain of P. chlororaphis or P. aureofaciens is cultivated at room temperature on tryptic soy agar (Difco) containing the same nitrate and ammonium amendments as the liquid medium. A starter tube containing 5 mL of the amended tryptic soy broth is inoculated with an individual colony and allowed to grow overnight on a reciprocal shaker. The 400-mL cultures are inoculated from the starter tube, and the media bottle tops are sealed tightly. The cultures are then grown for 6–10 days on a reciprocal shaker.

The organic nitrogen in the tryptic soy broth and the ammonium added to the culture medium provide nitrogen for assimilation, ensuring that the nitrate sample is not assimilated into bacterial biomass. Stoichiometric conversion of nitrate to nitrous oxide has been demonstrated previously for P. chlororaphis in tryptic soy broth without added ammonium,8,9 thus, our addition of ammonium is precautionary. The six-day incubation provides adequate time for complete consumption of the O₂ in the headspace and the amended nitrate. In some cases, most commonly for P. chlororaphis, a four- or five-day incubation is adequate.

**Conversion of Sample to Nitrous Oxide.** On the day of sample preparation, the culture is divided into 40-mL aliquots and centrifuged for 10 min at 7500g in a fixed-angle, refrigerated (18 °C) centrifuge. The supernatant medium is then decanted, and each cell pellet is resuspended in 4 mL of spent medium, representing a 10-fold concentration of cells. The concentrated cells are then aliquotted into 20-mL headspace vials (2 mL per vial), with each vial representing one analysis. The vials are capped with Teflon-backed silicone septa and crimp seals.

To remove the N₂O produced from the original 10 mM nitrate and to ensure anaerobic conditions, each sealed vial is purged at 10–20 mL/min for 2 h or more with N₂ gas. The purging gas is introduced through a 26-gauge needle, inserted through the septum so as to bubble the medium, and is vented through a 25-gauge needle that is inserted above the liquid level. At the end of the purging time, the vent needle is removed, followed immediately by the bubbling needle.

A water sample is then injected into each vial using a leak-tight syringe and a 25-gauge needle. The sample itself is not typically purged to remove N₂O or O₂, because no difference has been noted with the inclusion of this step, and this additional manipulation involves some risk of contamination. For samples of 4 mL or greater, a venting needle is placed through the septum of the headspace vial during the addition of the sample to prevent pressurization of the vial and subsequent loss of N₂O gas.

The volume of sample is adjusted to achieve a final sample size of 10–20 nmol N (5–10 nmol N₂O), which is optimal for our system. Smaller or larger amounts can also be used according to the specific capabilities of the mass spectrometer system.
however, the volume of sample should not be more than 5-fold greater than the volume of cell concentrate. Commonly, it is desirable to obtain a consistent final quantity of N₂O for both samples and standards in order to minimize uncertainties associated with any nonlinearity in the isotope-ratio mass spectrometer. After sample addition, the vials are incubated overnight to allow for complete conversion of nitrate to N₂O. The sample vials are stored inverted until the N₂O gas is extracted; this reduces any loss of N₂O through leaks that might be present in the seal.

After the overnight incubation, 0.1–0.2 mL of 10 N sodium hydroxide is injected into each headspace vial, which brings a seawater sample to a pH greater than 12, lyses the bacteria, and stops the reaction. In addition, the added base immobilizes the bulk of the CO₂ gas in the sample as dissolved inorganic carbon. The vapor pressure of N₂O is similar to that of CO₂ at most temperatures. Moreover, N₂O and CO₂ have identical molecular weights (44, 45, and 46), so CO₂ and N₂O interfere in the mass spectrometer. Accordingly, removal or separation of CO₂ from the N₂O sample is required prior to isotopic analysis. As described below, there are steps associated with the extraction and on-line purification that are designed to accomplish this; however, sequestration of the bulk of the CO₂ in the medium by the addition of sodium hydroxide is an important precursor to these on-line steps.

For natural-abundance isotope work, complete conversion of the nitrate sample to nitrous oxide is critical because of the inherent isotope fractionation of the process.13 With complete conversion, the nitrogen isotope mass balance between reactant and product prevents this inherent fractionation from being expressed in the isotope results. According to Christensen and Tiedje,9 the conversion of nitrate to N₂O is complete after 10 min for P. chloraraphis. Our data generally confirm this time scale for conversion by both P. chloraraphis and P. aerofaciens, with the conversion to N₂O close to completion after 30 min (Figure 1); however, for accurate isotopic analyses, we have found the requisite time for complete reduction to vary with the nitrate concentration and volume of the sample (longer for a lower nitrate concentration and larger sample volume) and the age of the bacterial culture (typically longer for an older culture). In our early experiments, the reaction was stopped sequentially as N₂O extractions were carried out, so that the incubation times for samples at the beginning and end of the run varied by as much as 6 h. Our data indicate no deleterious effects from an incubation time that is longer than necessary (Figure 1c), so we now add samples to prepared vials on one day, incubate the samples overnight, and analyze the product N₂O on the following day.

**Extraction and Isotopic Analysis of Nitrous Oxide.** Using a helium carrier gas, N₂O is stripped from each sample vial, purified, and analyzed for its isotopic composition using an isotope ratio mass spectrometer. This can be accomplished using published trace-gas methods14 or a derivative thereof. We have used two approaches. The first approach involves an off-line extraction of the N₂O from the sample vials, followed by on-line purification, cryogenic trapping, chromatographic separation from CO₂ using a Finnigan Precon system, and isotope-ratio analysis using a Finnigan MAT 252. The second approach uses a fully automated system for N₂O extraction, purification, and isotopic analysis using a modified Finnigan GasBench and DeltaPlus. The manual approach is described briefly below, and the automated system will be described elsewhere (D. M. Sigman and A. Hilkert, in preparation).

The manual N₂O extraction system (Figure 2) is composed of (i) a helium supply that is carried by 1/8-in. o.d. nylon tubing among the different components of the extraction system; (ii) a flow controller (0–100 mL/min); (iii) two ports for 25-gauge needles to strip gases out of the sample vial and transfer the carrier gas stream to the purification and sample collection traps; (iv) a gas drying bulb (i.e., “water trap”) filled with glass beads and immersed in a −70 °C alcohol bath; (v) a sample N₂O trap, which is a glass U-tube with a stopcock at both ends, ~50% filled with glass beads, and immersed in a liquid N₂ bath; and (vi) a manual on/off valve to switch among purging and extraction modes (Figure 2a,b, respectively). The system is purged with helium at 80 mL/min for 5 min prior to each extraction, with the manual valve switched to the open position to allow helium to purge both needle ports, the water trap, and the sample N₂O trap (Figure 2a). In expectation of sample extraction, the U-tube is placed in a liquid nitrogen bath. The flow rate is then reduced to 20 mL/min, and the needles are inserted into the sample vial, with the longer input needle immersed in the sample solution. The manual valve is then switched to the closed position, forcing the helium to flow through the sample vial, extracting the N₂O from the sample and transferring it to the U-tube (Figure 2b). Tests of the extraction system show that, with a helium flow rate of 20 mL/min, all of the N₂O in a 25-ml headspace vial is extracted within 15 min. The U-tube stopcocks are then closed, upstream stopcock first, the liquid nitrogen bath is removed, and the sample-bearing U-tube is replaced with a second U-tube for the next extraction. The manual valve is then switched to the open position, the flow is increased to 80 mL/min, and the needles are removed from the sample vial and placed in a water-filled beaker, thus returning the system to purging mode.

The U-tube holding the N₂O sample is placed in-line to a Finnigan MAT Precon device on-line to a Finnigan MAT 252 isotope ratio mass spectrometer.15 Several steps are accomplished in this device before the N₂O sample is introduced to the mass spectrometer: (i) the sample in helium carrier is sent through a chemical trap for water and carbon dioxide, (ii) the N₂O is cryogenically focused, and (iii) the resulting N₂O peak is chromatographically separated from any remaining carbon dioxide. In the mass spectrometer, measurements at the massess 44, 45, and 46 allow for coupled measurement of the 15N/14N and 18O/16O ratios of N₂O, once a correction is made for the contribution of 17O to mass 45.15

**Isotopic References.** Individual sample analyses are referenced against automated injections of N₂O from a gas cylinder; however, the N₂O cylinder is not used as the absolute reference. Rather, each batch of samples includes replicates of an internationally recognized nitrate standard (IAEA-N3) that are used to calibrate isotopic ratios to that of air N₂. IAEA-N3 has an assigned δ¹⁵N of 4.7‰ versus air N₂ and a reported δ¹⁸O of 22.7‰ versus

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Standard Mean Ocean Water (SMOW) is preferable to the use of the N$_2$O reference gas as the means of calibration, because it accounts for possible changes in any aspect of the analysis, including (i) changes in the size or isotopic composition of the bacterial N$_2$O blank; (ii) changes in instrument linearity, which will affect the comparison of reference N$_2$O pulses to the sample N$_2$O peak; (iii) drift in the isotopic composition of the reference gas, which has been shown to occur in some systems. In any case, nitrate standards must be run with each batch of samples to confirm expected performance of the analysis.
RESULTS AND DISCUSSION
Precision within and between Different Runs. Analyses of the isotopic standard IAEA-N3, when compared with automated injections from the N2O reference tank, yield a standard deviation of 0.2% or better for a given batch of analyses and typically result in means that are nearly indistinguishable among different days and the two bacterial strains (Table 1). As described above, this nitrate standard, not the reference N2O tank, is used to calibrate our isotopic (δ) scale. Therefore, excellent long-term accuracy relative to the reference tank is not required. Nevertheless, the observed stability indicates that both the bacterial production of N2O and the isotopic analysis of this N2O are inherently reproducible at the natural-abundance level.

Comparison of Nitrate Reference Materials. Two in-house nitrate standard salts were combusted to N2 and analyzed using an elemental analyzer/mass spectrometer system in the lab of M. A. Altabet. These same two standards, dissolved and diluted to 20 μM in deionized water and Sargasso Sea surface water, were also analyzed using the denitrifier method (Table 2). The comparison shows that the denitrifier method reproduces the δ15N difference between the two standards as measured by direct combustion. That our measured δ15N difference between the two nitrate salts is 0.1–0.2% greater than that measured by direct combustion does not concern us greatly. Rather, we would be concerned if our measurements underestimated the isotopic difference between the two salts, which might occur if an unknown

![Diagram of N2O extraction system](image)

**Figure 2.** Manual N2O extraction system in (a) purging mode and (b) N2O extraction mode (see text).

**Table 1. Replicates of Reference Nitrate IAEA-N3**

<table>
<thead>
<tr>
<th>Strain</th>
<th>P. aureofaciens</th>
<th>P. aureofaciens</th>
<th>P. chlororaphis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date</td>
<td>12/29/99</td>
<td>1/3/00</td>
<td>1/7/00</td>
</tr>
<tr>
<td>δ15N‰ n = 3b</td>
<td>5.04</td>
<td>5.12</td>
<td>4.98</td>
</tr>
<tr>
<td>Std dev. %n = 3</td>
<td>0.14</td>
<td>0.02</td>
<td>0.05</td>
</tr>
</tbody>
</table>

a Three batches IAEA-N3 were processed and analyzed on different days. The standard was diluted to 20 μM in deionized water. Each analysis was of 20 nmol of nitrate-N. Isotopic ratios are relative to automated injections from a reference N2O tank.

**Table 2. Isotopic Difference between In-House Nitrate Standards from Independent Methods**

<table>
<thead>
<tr>
<th>Method</th>
<th>Deionized Water</th>
<th>Sargasso Sea Water</th>
<th>Combustion to N2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denitrifier method</td>
<td>0.73</td>
<td>0.63</td>
<td>0.55</td>
</tr>
<tr>
<td>Combustion to N2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Analyses by the denitrifier method were of 20 nmol of nitrate-N from 20 μM solutions, and the samples analyzed by on-line combustion were of 2 μmol nitrate-N pipetted from 1 mM stock solutions. The δ15N difference was measured between triplicates. The standards in deionized water and Sargasso Sea surface water were prepared and analyzed on different days.
### Table 3. Isotopic Difference between Reference Materials

<table>
<thead>
<tr>
<th></th>
<th>USGS-32</th>
<th>IAEA-N3</th>
<th>date of analysis&lt;sup&gt;a&lt;/sup&gt;</th>
<th>assigned values</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta^{15}N$, %</td>
<td>170.94</td>
<td>171.08</td>
<td>12/17/99</td>
<td>12/29/99</td>
</tr>
<tr>
<td>$\Delta^{15}N$, %</td>
<td>174.5–175.3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> The $\Delta^{15}N$ difference was measured between triplicates in two separate experiments run on different days.

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The background nitrogen pool were diluting the sample $N_2O$ pool generated from our nitrate standards.

Two internationally recognized standards IAEA-N3, with $\delta^{15}N$ of 4.7‰ vs air<sup>16</sup> and USGS-32, with a $\delta^{15}N$ of 179.2 to 180‰<sup>16</sup> were also diluted to 20 $\mu M$ in deionized water and analyzed. The $\delta^{15}N$ difference between USGS-32 and IAEA-N3 from our analyses is compared with the difference between the assigned values of these nitrate salts (Table 3). Our 4‰ underestimation of the $\delta^{15}N$ difference between the two standards is reproducible and is of the amplitude expected as a result of our blank, which is 2.5‰ of the sample size in this case (0.5 nmol of N of blank compared with 20 nmol of added nitrate-N; see discussion of blank results below).

**Intercalibration.** To provide a more functional comparison of our method with previously published methods, we analyzed splits of groundwater and precipitation samples of nitrate and nitrite analyzed previously in the USGS Reston Stable Isotope Laboratory using established methods for freshwaters<sup>17,18</sup> (Figure 3). The samples were provided either as dried salts or water samples with millimolar nitrate or nitrite; all were subsequently diluted to ~20 $\mu M$ solutions using deionized water. Analyses were of 10 nmol of nitrate- or nitrite-N (0.5 mL of our 20 $\mu M$ solutions).

Agreement is excellent for most groundwater samples (Figure 3, black symbols); however, for samples with very high $\delta^{15}N$ values, our method tends to underestimate this value, for example, by 2‰ for a sample with a “true” $\delta^{15}N$ of 104‰. Conversely, a nitrite sample with a very low $\delta^{15}N$ of ~80‰ is overestimated by ~2‰. These discrepancies are consistent with dilution by the nitrogen blank of our method. This blank is 0.5 nmol of N (see below), which represents 5% of the sample if 10 nmol of nitrate is added and would be expected to cause a ~5‰ error for nitrate that is 100‰ different from the isotopic standard, assuming that the $\delta^{15}N$ of the blank is the same as the nitrate added to the growth medium (~5‰ seen dashed line in Figure 3).

In the case of the precipitation samples, which have very high $\delta^{18}O$ values (60–80‰ vs SMOW<sup>19</sup>), we tend to overestimate the $\delta^{15}N$ by 1–2‰ with these samples clearly falling off the relatively tight agreement line observed for other sample types (Figure 3, open squares). This error probably involves our correction of the mass 45 signal for the contribution from $^{14}N_2^{17}O$. Although we have made the standard assumption of a mass-dependent $^{14}O$/ $^{17}O$/$^{18}O$ relationship<sup>15</sup>, nitrate in precipitation may well be impacted by mass-independent reactions through exchange with ozone, which shows strong mass-independence in its $^{18}O$/ $^{16}O$ and $^{17}O$/ $^{16}O$ differences from SMOW.<sup>20</sup> Indeed, our calibration study suggests that the $\delta^{17}O$ and $\delta^{18}O$ of atmospheric nitrate are dominated by mass-independent fractionation,<sup>21</sup> a suggestion that is confirmed by measurement of the $\delta^{17}O$ of $O_2$ derived from the stepwise combustion of atmospheric nitrate.<sup>21</sup> The comparison of $N_2O$- and $N_2$-based isotopic analyses of nitrate provides a novel approach for quantifying the $\delta^{15}O$ of nitrate (M. Galanter et al., in preparation).

**Blanks and the Effect of Nitrate Concentration.** The blank size was investigated by the analysis of “nitrate-free” water, both deionized water and Sargasso Sea surface water (Figure 4). The cause of the increase in blank size with increasing sample volume is not yet clear. Possibilities considered so far include (i) $N_2O$ dissolved in the water at roughly twice saturation with the atmosphere, (ii) a nitrate concentration of ~30 nM in both the deionized water and our stored Sargasso Sea water, or (iii) the release of strongly sorbed $N_2O$ from the bacterial biomass, occurring to greater degrees at greater dilutions. Dissolved $N_2O$ can be rejected as the source of the volume dependence in the blank on the basis of blank tests that show no significant reduction in blank size after purging the nitrate-free water with $N_2$ gas (data not shown). We have not yet ruled out an important contribution from residual nitrate in the water. However, in support of the third option, if bacterial concentrate is purged for only 20 min before the sample is added (rather than the standard time of 2 h), the increase in blank with volume is much greater, such that only $N_2O$ desorption from the bacterial concentrate can explain the blank size for large sample volumes (data not shown). It seems reasonable to assume that this process is occurring, albeit to a greatly diminished degree, when the bacterial concentrates are purged for the standard time of 2 h.

Because the blank size is highly reproducible for a given batch of samples, an offset correction is made for the isotopic effect of the blank by shifting the $\delta^{15}N$ of the entire sample batch such that the replicates of IAEA-N3 have a mean $\delta^{15}N$ of 4.7‰ vs air. This is not a theoretically complete correction, in that the effect of the blank will depend on the size of the blank relative to each sample and the $\delta^{15}N$ difference between the blank and each sample. However, the effect of the blank on the measured isotopic difference between any two samples is minor (Tables 2 and 3, Figure 3), presumably because the size of the blank is small, typically ~0.5 nmol of N, as compared to a sample of 10–20 nmol of N, and because the $\delta^{15}N$ of the blank appears to be ~5‰ (data not shown), which is indistinguishable from the $\delta^{15}N$ of the nitrate on which the denitrifier culture is grown and is not very different from the $\delta^{15}N$ of nitrate in most environments.

The major concern with the blank is its apparent increase with the volume of sample added (Figure 4). Because lower nitrate concentration samples require the addition of a larger sample volume, the blank could cause a dependence of the measured $\delta^{15}N$ on the concentration of the samples. Given that the blank is always less than 10% of the sample, its isotopic effect should be small even in the case that it is very isotopically different from the samples. For instance, if the blank is assumed to be 10%...
different from a given nitrate sample and the volume dependence of the blank is taken from Figure 4, a change from 20 to 1 mM the concentration of that nitrate in solution will cause only a 0.25‰ change in the δ¹⁵N of the nitrate generated from that nitrate. Even this small concentration dependence in δ¹⁵N is not evident in a number of concentration-range experiments performed with nitrate standard IAEA-N3 (Figure 5). Under appropriate conditions, using either P. chlororaphis or P. aureofaciens, the same δ¹⁵N value is measured for an isotopic standard down to nitrate concentrations of 1 mM. This supports the other indications that the δ¹⁵N of the blank is typically not very different from the δ¹⁵N of IAEA-N3 (4.7‰) and, thus, is also not very different from most samples of natural nitrate. Nevertheless, the volume dependence of the blank remains a concern, and ongoing work in our laboratory focuses on removing it and reducing the blank size in general.

Previously published methods for nitrogen isotopic analysis of seawater nitrate have a significant blank associated with dissolved organic nitrogen, for which it is difficult to correct because of its variation with sample type.²,⁶ In the denitrifier method, the amount and lability of dissolved organic nitrogen in the denitrifier culture medium is orders of magnitude higher than in any foreseeable natural sample. Thus, the medium dominates the dissolved organic nitrogen blank, so that this blank will not vary with sample type or volume. Moreover, our tests indicate that the dissolved organic nitrogen blank is small, less than 0.5 nmol of N (Figure 4).

Application to Marine Sediment Porewaters. To give a sense of the new studies that the denitrifier method makes possible, we report the first analyses of nitrate δ¹⁵N from marine sediment porewaters, a completely novel measurement that relies on the analytical advances associated with the denitrifier method. These porewater measurements require three improvements from previously available methods that are achieved by the denitrifier method. First, the isotopic analysis of small (i.e., nanomole) amounts of nitrate is necessary, because only milliliters of porewater can be collected in most cases. Second, the isotopic analysis of samples with low nitrate concentrations (e.g., 1 mM) is not evident in a number of concentration-range experiments performed with nitrate standard IAEA-N3 (Figure 5). Under appropriate conditions, using either P. chlororaphis or P. aureofaciens, the same δ¹⁵N value is measured for an isotopic standard down to nitrate concentrations of 1 mM. This supports the other indications that the δ¹⁵N of the blank is typically not very different from the δ¹⁵N of IAEA-N3 (4.7‰) and, thus, is also not very different from most samples of natural nitrate. Nevertheless, the volume dependence of the blank remains a concern, and ongoing work in our laboratory focuses on removing it and reducing the blank size in general.

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in seawater is necessary, because nitrate is consumed by denitrification in the deep porewaters. Third, given the high and variable concentration of dissolved organic matter in marine sediments, the analyses must not be contaminated by the dissolved organic nitrogen in porewaters.

The porewaters were collected from a multicore raised from 3418 m on the Carolina Slope in the western Atlantic. The nitrate concentration profile from this site consists of a shallow maximum and a subsequent decline with depth, typical of porewater profiles in regions of suboxic diagenesis (Figure 6). The nitrate maximum in the shallow sediment is generated from the breakdown of organic nitrogen and its oxidation to nitrate, and the downward nitrate decrease results from consumption by denitrification in the deeper sediments, where oxygen has been nearly completely consumed. The single-point minima in nitrate concentration have been reproduced and may be the result of burrowing and the influx of bottom water (with a \([\text{NO}_3^-] \approx 17 \text{ M}\)) or the in situ consumption of nitrate by denitrifying microzones.

The major feature of the nitrate \(\delta^{15}N\) profile is the downward \(\delta^{15}N\) increase associated with nitrate consumption by sedimentary denitrification (Figure 6a). The nitrogen isotope effect for denitrification that is estimated from the data using the Rayleigh model (consumption of a closed nitrate pool by a process with a constant isotope effect) is \(6.7\%\) (Figure 6b). This estimate is much lower than those of culture and oceanic water column studies, which typically indicate a nitrogen isotope effect for denitrification of 20–30%. The discrepancy is not surprising, in that the sediment porewaters are an open system, with gross diffusion in all directions and net nitrate diffusion downward from shallower porewaters, whereas the Rayleigh model for nitrate consumption assumes a closed system. The low Rayleigh-based estimate for the isotope effect, therefore, is probably due to the combined effect of a high degree of nitrate consumption at depth and the diffusion of nitrate between high- and low-\([\text{NO}_3^-]\) porewaters, which tends to reduce the isotopic enrichment from that generated in a closed system. This effect has been explored for N isotopes in different systems and for sulfur isotopes in sedimentary porewaters.

Figure 5. \(\delta^{15}N\) and \(N_2O\) yields from concentration series of additions of reference nitrate IAEA-N3, for (a,b) \(P.\) aureofaciens and (c,d) \(P.\) chlororaphis after dilution in (a, b, c) deionized water and (b, c, d) Sargasso Sea surface water. To make the panels comparable, the \(\delta^{15}N\) value is referenced to 4.7‰ vs air \(N_2\) for the 20 \(\mu M\) nitrate standards. The yields are given in terms of the mass-44 peak area. In (a), (b), and (d), 10 nmols of nitrate-N were processed per analysis, and 20 nmol of N was processed in (c), explaining the large difference in signal. The smaller differences in signal among the experiments are most likely due to changes in the conditions of the helium flow or the mass spectrometer.

Because of the under-expression of the isotope effect for denitrification (and the possible isotopic effect of nitrification), the $\delta^{15}$N of nitrate in the shallowest porewaters at this site does not bear a significant isotopic imprint from exchange with the deeper, partially denitrified porewaters. Because the overlying bottom water exchanges with the shallow porewaters, the $\delta^{15}$N of bottom water nitrate (4.8‰) is not greatly affected by sedimentary denitrification in this region. These results extend the set of sedimentary porewater environments in which the isotope effect of denitrification has been shown to be greatly under-expressed.29 A more complete discussion of these results will be provided elsewhere (D. M. Sigman and D. C. McCorkle, in preparation).

CONCLUSIONS

The denitrifier method is a fully operational method for the nitrogen isotopic analysis of nitrate. Many of the critical needs of the isotope community (e.g., low sample size requirement, reduction in the required sample size and nitrate concentration, and absence of a large or variable blank from dissolved organic nitrogen) have already been realized, and continued work will allow the analysis of smaller samples with still lower nitrate concentrations. Various approaches for the conversion of nitrate to nitrous oxide have been recognized,33,34 and it seems likely that some of these approaches can be adapted for the isotopic analysis of nitrate at natural-abundance levels. The success of the denitrifier method provides proof of concept for other potential nanomole-level, N$_2$O-based methods of nitrate isotopic analysis.

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