# Chemical Conversion of Nitrate and Nitrite to Nitrous Oxide for Nitrogen and Oxygen Isotopic Analysis in Freshwater and Seawater

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# Chemical Conversion of Nitrate and Nitrite to Nitrous Oxide for Nitrogen and Oxygen Isotopic Analysis in Freshwater and Seawater

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**We present a novel method for nitrogen and oxygen natural isotopic abundance analysis of nitrate and nitrite of seawater and freshwater at environmental concentrations. The method involves the reduction of nitrate to nitrite using spongy cadmium with further reduction to nitrous oxide using sodium azide in an acetic acid buffer. For separate nitrite analysis, the cadmium reduction step is simply bypassed. Nitrous oxide is purged from the water sample and trapped cryogenically using an automated system with subsequent release into a gas chromatography column. The isolated nitrous oxide is then analyzed on a continuous flow isotope ratio mass spectrometer via an open split. This paper describes the basic protocol and reaction conditions required to obtain reproducible natural abundance level nitrogen and oxygen isotopic ratios from nitrate, nitrite, or both, and the results obtained to support these conclusions. A standard deviation less than 0.2‰ for nitrogen and 0.5‰ for oxygen was found for nitrate samples ranging in concentration from 40 to 0.5 μM (better for nitrite), with a blank of 2 nmol for 50-mL samples. Nitrogen and oxygen isotopic fractionation and oxygen atom exchange were consistent within each batch of analysis. There was no interference from any seawater matrixes. Only one other method published to date can measure the nitrate oxygen isotopic abundance in seawater and none that do so for nitrite alone in the presence of nitrate. This method may prove to be simpler, faster, and obtain isotopic information for lower concentrations of nitrate and nitrite than other methods.**

Several approaches to analyze nitrogen and oxygen isotopic ratios in nitrate have been developed over the past few decades. An adaptation of the ammonia diffusion method,[1,2] solid-phase extraction of nitrate as 1-phenylazo-2-naphthol[3] and bacterial conversion of nitrate to nitrous oxide[4] are methods that have been successful for measuring natural abundance levels of nitrogen isotopes of nitrate in freshwater or seawater. Pyrolytic methods have been useful for freshwater samples[5,6,21] and have been critical for determining the references $\delta^{18}O$ of standard materials, but bacterial conversion of nitrate to nitrous oxide is the only published method successful in measuring the oxygen isotopic levels of nitrate in seawater. Bacterial conversion of nitrate to nitrous oxide is the only published method successful in measuring the oxygen isotopic levels of nitrate in seawater.[1] No method has been developed to measure nitrite oxygen and nitrogen isotopic ratios at natural abundance levels separately from nitrate.

Several advances in methodology have permitted the success of this method. The development of automated continuous flow, purge-and-trap analysis has made the rapid isolation and analysis of nitrous oxide possible and has drastically increased sample throughput.[1] References provided by on-line pyrolysis of nitrate and nitrite salts have also permitted the development of a method that can provide reliable isotopic data simply by calibration with these references.[17]

We have developed methodology for the determination of the $\delta^{15}N$ and $\delta^{18}O$ of nitrate or nitrite based on the reaction between nitrite and azide to form $N_2O$:

$$HNO_2 + HN_3 \rightarrow N_2O + H_2O + N_2$$

The reaction mechanisms and kinetics were described extensively in a series of papers by Bunton and Stedman in the 1950s.[8−16] Previous work by Shearer and Kohl[19] as well as Garber and

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Hollocher[20] utilized the reaction between nitrite and azide to produce nitrous oxide for the analysis of $\delta^{15}N$ and $\delta^{18}O$ of nitrite in denitrifying bacteria experiments. They emphasized necessary conditions previously identified by Bunton and Stedman and verified by us with respect to pH range and halide concentration. In optimizing this method for reliable and routine $\delta^{15}N$ and $\delta^{18}O$ determination of nitrite and nitrate at environmental concentrations in seawater as well as freshwater, we have found two other conditions previously identified, high azide concentration and anaerobicity, to be unnecessary with our analytical procedures. In the present article, the $\delta^{15}N$ and $\delta^{18}O$ values of the product $N_2O$ were determined using automated continuous flow IRMS equipment similar to that previously utilized for nitrous oxide isotopic analysis.[1] Spongy cadmium, commonly used for spectrometric determination of nitrate concentrations, was used to convert nitrate to nitrite.[7] The sodium azide step was then used to convert nitrite to $N_2O$. Alternatively, nitrite can be measured alone by reduction using just the sodium azide step.

In this paper, we describe the performance characteristics of the new methods under various conditions in seawater and freshwater. Reagent concentration, pH, and reaction time were varied to optimize the reaction between nitrite and sodium azide. The isotopic composition of $N_2O$ was measured over a range of $^{15}N$ and $^{18}O$ enrichments in nitrite and nitrate to determine the performance of the method, as well as varying the $\delta^{18}O$ of the substrate water. The automated purge-and-trap sequence was optimized for maximal yield, minimal interference from contaminants, and minimal run time.

**Reaction Mechanism of Nitrite and Azide**. The best proposed mechanism for the reaction between nitrite and azide at pH >2 is the formation of the nitrous acidium ion ($H_2NO_2^+$) followed by reaction with the azide ion:[15]

$$NO_2^- + H^+ \leftrightarrow HNO_2$$

$$HNO_2 + H_2O \leftrightarrow H_2NO_2^+ + OH^-$$

$$H_2NO_2^+ + N_3^- \xrightarrow{\text{slow}} N_3NO + H_2O \xrightarrow{\text{fast}} N_2O + N_2 \quad (1)$$

Under highly acidic conditions ($[H^+]$ > 0.05 M), the nitrous acidium ion reacts with hydrazoic acid:[14]

$$H_2NO_2^+ + HN_3 \xrightarrow{\text{slow}} N_3NO + H_2O + H^+ \xrightarrow{\text{fast}} N_2O + N_2 \quad (2)$$

Both reactions rely on the formation of the nitrous acidium ion. Therefore, both are pH dependent and increase in rate as pH is decreased.

In the presence of chloride and bromide ions, the rate is increased, and the proposed mechanism for this catalysis is[15]

$$H_2NO_2^+ + Cl^- \xrightarrow{\text{slow}} NOCl + H_2O$$

$$NOCl + N_3^- \xrightarrow{\text{fast}} N_3NO + Cl^- \xrightarrow{\text{fast}} N_2O + N_2 \quad (3)$$

The rate is therefore dependent on pH, nitrous acid concentration, and halide concentration. Stedman found the reaction to follow the rate equation[15]

$$v = k[HNO_2][H^+]$$

The following rates for nitrous oxide formation were found:[16]

$k = 260\ s^{-1}\,mol^{-1}\,L$ in azide and hydrochloric acid buffer

$k = 230\ s^{-1}\,mol^{-1}\,L$ in excess perchloric acid

Although these rates were determined using nitrite concentrations much higher than those used in this study, it was expected that the relative reaction rates would remain the same according to changes in pH.

**Nitrogen Isotopic Composition of Produced $N_2O$.** Assuming the reaction goes to completion, the resulting nitrogen isotopic composition of the product nitrous oxide should be the average of the $\delta^{15}N$ values of the nitrite and azide since each contributes one N atom. The expected slope of a plot of $\delta^{15}N$ of standard nitrite versus measured $\delta^{15}N$ of the produced nitrous oxide would be 0.5. A lower than expected intercept could indicate a preference of $^{14}N$ of azide in the reaction between nitrite and azide, resulting in a depleted $\delta^{15}N$ product, $N_2O$, and an enrichment of $^{15}N$ in the second product, $N_2$. This is possible since only a small fraction of the azide is converted to $N_2O$ whereas the conversion of the nitrite is quantitative.

**Oxygen Isotopic Composition of Produced $N_2O$.** As shown below, the oxygen isotopic composition of nitrous oxide produced from the reaction between nitrite and azide reflects that of the starting nitrite, oxygen exchange with water during the reaction, and isotopic fractionation during the cadmium or azide reduction. The slope of a plot of $\delta^{18}O$ of nitrous oxide versus $\delta^{18}O$ of the nitrite that the nitrous oxide is derived from should have a slope of 1 if no oxygen exchange occurs with water and an intercept associated with isotopic fractionation. The slope would be zero if complete exchange with water occurred.

The amount of oxygen exchange between nitrite and water is highly dependent upon reaction conditions. It was expected that most of the oxygen exchange occurred during the nitrite to nitrous oxide step because initial results showed that variations in pH during the azide−nitrite reaction produced large variations in oxygen exchange. An examination of the reaction mechanism was used to find the conditions that would minimize the exchange of oxygen with water in order to maximize the precision and consistency of the $\delta^{18}O$ sample analysis.

The most likely mechanism for oxygen isotope exchange during the azide reaction is the direct attack of the nitrous acidium ion on water:[15]

$$H_2NO_2^+ + H_2{}^{18}O \leftrightarrow H_2NO^{18}O^+ + H_2O \quad (4)$$

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The oxygen exchange rate equation is best followed by

$$v = k[HNO_2][H^+]$$

Stedman found the following rate for oxygen exchange between nitrous acid and water:[14]

$$k = 220\ s^{-1}\ mol^{-1}\ L$$

The exchange rate and the two reaction rates can be directly compared because they all follow the same rate equation. At a very low pH, the oxygen exchange rate ($k = 230\ s^{-1}\ mol^{-1}\ L$) is not significantly different from the rate of production of nitrous oxide ($k = 220\ s^{-1}\ mol^{-1}\ L$) via the nitrous acidium ion and hydrazoic acid, and it was predicted that some amount of oxygen exchange would occur. At a pH greater than 2, the reaction rate of the azide ion and the nitrous acidium ion ($k = 260\ s^{-1}\ mol^{-1}\ L$) is significantly higher than the oxygen exchange rate, and it was expected that there would be less oxygen exchange.

The concentration of chloride or bromide ions also has an important effect on the reaction rate and oxygen isotope exchange rate. These halides are reported to increase the rate of reaction as seen in reaction 3 and, at the same time, increase the rate of oxygen exchange between $H_2O$ and $HNO_2$.[16] This is because halides may introduce a new oxygen exchange path:

$$NOX + H_2O \leftrightarrow H_2NO_2^+ + X^- \qquad (5)$$

Accurate rate determinations were not obtained in previous papers, particularly for our reaction conditions, which were suitable for natural concentration levels of nitrate and nitrite. It was unknown if halides would be beneficial to oxygen isotopic analysis through reduction of oxygen isotopic exchange.

The amount of oxygen exchange of the produced nitrous oxide is dependent on a competition between the above five reactions. In acidic conditions, reaction 2 dominates, and the rate of the chemical reaction is near to the rate of the oxygen exchange reaction. In azide buffers, the rate of the chemical reaction is greater than the rate of oxygen exchange, and reaction 1 dominates. From this information, we can predict that increasing the pH will decrease the observed oxygen exchange in the final product $N_2O$. Since both the oxygen exchange and nitrous oxide formation rates are dependent on hydrogen ion concentration, the overall reaction rate should decrease with increasing pH.

## METHODS

**Reagents.** Spongy cadmium was made by adding zinc sticks to a 20 wt % solution of cadmium chloride in deionized water (DIW). The reduced cadmium crystals were scraped off the zinc sticks into a separate beaker in roughly 15-min intervals to prevent the cadmium from becoming too thick. Once the desired amount of cadmium was obtained, it was broken into small pieces before rinsing with 6 M HCl for ~1 min. The cadmium was then rinsed repeatedly with DIW until the pH was neutral (typically 10 rinses). It was then stored in DIW before use and used within several hours. After use, the cadmium was collected and the acid wash and rinse steps were repeated for reuse. The cadmium was reused in this manner until it dissolved completely from the repeated acid rinses.

The azide and acetic acid buffer was prepared by combining a 1:1 by volume mixture of 20% acetic acid and 2 M sodium azide. The buffer was then purged with He at 40 mL/min for 10 min to remove any $N_2O$ produced from nitrite contained in the reagents. This solution was prepared on the day it was to be used. All reactions were performed in a fume hood due to the volatility and toxicity of $HN_3$, which is the dominant species below pH 4.6. Solutions were made basic (pH >10) before analysis on the purge-and-trap system to prevent $HN_3$ escaping into the laboratory or the nitrous oxide trapping system. $HN_3$ is extremely toxic and volatile, so it must be handled carefully in a fume hood at all times. $HN_3$ is also explosive in concentrated solutions; therefore, it is best to keep volumes small and make sure all solutions are made basic immediately following the completion of the reaction.

**Conversion of Sample Nitrate to Nitrite.** The 50-mL water samples were placed in 60-mL vials with Teflon-lined caps. One gram of spongy cadmium was added followed by 1.0 mL of 1 M imidazole solution with a resultant pH close to 9. For freshwater samples, sodium chloride was added to make a final concentration of 0.5 M. The samples were then shaken overnight on a horizontal shaker at a rate of ~2 cycles/s. Alternatively, samples of 5 mL or less could be placed in a sonicator bath for 1 h with 0.3 g of spongy cadmium. The samples were then placed in a centrifuge at 1000 rpm for 10 min and decanted into fresh 60-mL vials with Teflon-lined septa.

**Conversion of Nitrite to Nitrous Oxide.** The vials were capped tightly with Teflon-lined septa, and 2 mL of the azide/acetic acid buffer was added to each vial via a syringe and shaken vigorously. After 15 min, the solution was made basic with a syringe addition of 1.0 mL of 6 M NaOH and shaken. All reactions were performed at room temperature.

**Analysis of Nitrous Oxide.** Samples were run on an automated purge-and-trap system prior to elution into a Finnigan MAT 251. Additional modifications of the mass spectrometer include differential pumping of the flight tube and updated electronics (developed by Clive Workman of Instrument Sciences). The schematics of the automated purge-and-trap system was similar to that used and described in detail by Casciotti et al.[1] A large-volume autosampler was used to purge each sample prior to trapping.

The sample was purged at a helium flow rate of 30 mL/min and sent through a Nafion drier, a magnesium perchlorate and Carbosorb trap, and frozen in a liquid nitrogen trap (double-layered loops of 0.4-mm-i.d. stainless steel, 12 turns of 1-in. diameter). After a 500-s purge time, the flow was switched to 3 mL/min He through the trap, and the trap was released from the liquid nitrogen. The sample was then diverted to another trap made of a single loop of 0.4-mm-i.d. stainless steel immersed in liquid nitrogen. After 80 s, the loop was lifted from the trap and thawed onto a 530 $\mu$m × 30 m GS-Q GC column held at room temperature, where the $N_2O$ was separated from any remaining $CO_2$ and oxygen. The GC stream was then sent through a cold bath (223 K, coldfinger immersed in ethanol) to remove any trace amounts of water or organic contaminants. The sample entered the mass spectrometer via a capillary from an open split directly

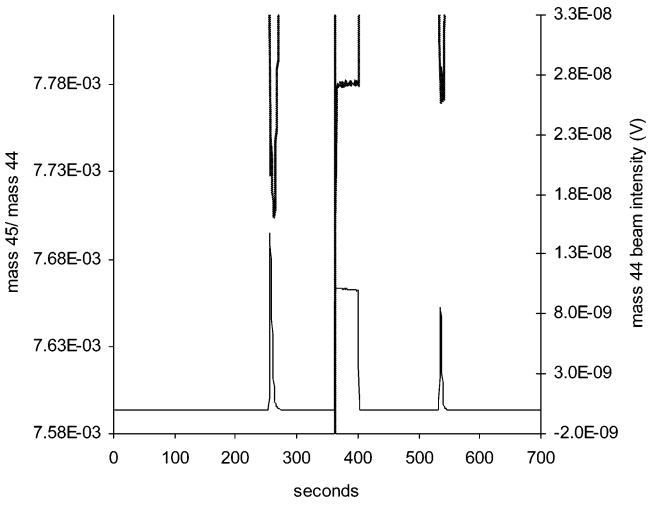


**Figure 1.** Typical run showing mass 44 beam intensity (solid line) and mass ratio 45/44 (dotted line). The first peak is nitrous oxide from the sample separated from carbon dioxide (carbon dioxide peak is too small to be visible in this plot: separation from nitrous oxide is ~22 s). The second peak is a nitrous oxide reference gas injected at the split inlet. The third peak is a 1% nitrous oxide in helium loop injected prior to the second liquid nitrogen focusing trap and was used to compare the calculation of a natural peak shaped standard gas injection to that of a standard injected via the open split.

to the ion source. Total run time was 700 s/sample. Each sample was accompanied by a direct inlet injection of pure $N_2O$ as well as a standard gas injection loop of 1% $N_2O$ in helium.

A typical run is shown in Figure 1. The first peak is the sample (nitrous oxide produced from nitrite or reduced nitrate to nitrite, purged, and trapped from the vial). The second peak is the pure nitrous oxide reference gas injected at the split inlet. The third peak is a 1% nitrous oxide in helium reference gas injected through a loop, trapped in the low-flow liquid nitrogen trap, and released through the GC column and into the mass spectrometer. Data were collected throughout the run for masses 44, 45, and 46 and integrated every 0.5 s. The area under each peak was calculated for each mass.

**Calculation of the True $\delta^{18}O$ for Nitrate and Nitrite Relative to VSMOW.** The true $\delta^{18}O$ of nitrate and nitrite samples were calculated by comparison to nitrate samples of known $\delta^{18}O$ and a nitrous oxide standard gas. Sample nitrous oxide was directly compared to the pure nitrous oxide injected with the accompanying sample:

$$\delta^{18}O_{sample} = (R_{sample} - R_{standard})/R_{standard} \times 1000$$

where $R$ is the ratio of the areas of masses 46/44. It was assumed that the nitrogen and oxygen isotopic values of the nitrous oxide standard gas did not change over time and was standardized against the nitrate and nitrite standards after reduction to nitrous oxide. Each sample and standard originating from the vials was compared directly to the pure nitrous oxide gas, and then the obtained values of the samples were compared to those of the standards. Nitrate samples were also corrected for the oxygen exchange that occurs between the sample and water during conversion to nitrous oxide and isotopic fractionation due to oxygen removal. This was done by dividing the $\delta^{18}O$ value of the sample by the slope of the curve made by nitrate standards of known $\delta^{18}O$:

$$\text{adjusted } \delta^{18}O_{\text{nitrate or nitrite}} = (\delta^{18}O_{\text{nitrous oxide}} - b)/m_{\text{standards}}$$

where $b$ is the $y$ intercept of the standards and $m_{standards}$ is the actual slope obtained by the standards, all reported relative to VSMOW.

When the $\delta^{18}O$ of the water used to make the standards was different from the $\delta^{18}O$ of the sample water, a correction for the oxygen exchange (19% for data in Figure 5) between the $\delta^{18}O$ of nitrate and water during the reduction to nitrous oxide needed to be added to the above equation:

$$\text{adj } \delta^{18}O_{\text{nitrate or nitrite}} = (\delta^{18}O_{\text{nitrous oxide}} - b)/m_{\text{standards}} - (1 - m_{\text{standards}})(\delta^{18}O_{H_2O\ \text{Sam.}} - \delta^{18}O_{H_2O\ \text{Std}})$$

where $(\delta^{18}O_{H_2O\ Sam} - \delta^{18}O_{H_2O\ Std})$ are the oxygen isotopic values of the water in which the nitrate samples or standards are dissolved relative to VSMOW. In ocean waters, where there is little change in the $\delta^{18}O$ of water, the standards are made in nitrate- and nitrite-depleted ocean water, and the above equation is not necessary.

**Calculation of the True $\delta^{15}N$ for Nitrate and Nitrite.** The initial calculation of the $\delta^{15}N$ of nitrate and nitrite nitrogen isotopes is similar to that of oxygen isotopes. The distinction is that there is an interference from $NN^{17}O$, which is the same mass[45] as $^{15}NNO$. In most cases, this $^{17}O$ signal is proportional to $^{18}O$ in nitrate and nitrite (mass-dependent isotope fractionation), and a correction is made (see Supporting Information).

The calculation of the sample $\delta^{15}N$ is performed starting with the direct comparison to the standard nitrous oxide:

$$\delta^{45}N_2O_{sample} = (R_{sample} - R_{standard})/R_{standard} \times 1000$$

where $R$ is the ratio of the areas of masses 45/44. Next is a $^{17}O$ correction assuming mass-dependent isotope fractionation:

$$\delta^{15}N_{sample} = \delta^{45}N_2O_{sample}[1 + {}^{17}R_{std}/(2^{15}R_{std})] - \delta^{17}O[{}^{17}R_{std}/(2^{15}R_{std})]$$

where ${}^{17}R_{std} = 0.037\ 99$, ${}^{15}R_{std} = 0.367\ 65$, and $\delta^{17}O = 0.52\ \delta^{18}O$. Then the adjustment is performed for the slope and intercept differences between the expected and actual values:

$$\text{adjusted } \delta^{15}N_{\text{nitrate or nitrite}} = (\delta^{15}N_{\text{nitrous oxide}} - b)/m_{\text{standards}}$$

where $b$ is the expected $y$ intercept of the standards and $m_{standards}$ is the actual slope obtained by the standards.

From the net reaction of nitrite with azide, we would expect to see an isotopic dilution of half the nitrite nitrogen, with one nitrogen originating from nitrite and the other from azide:

$$HNO_2 + HN_3 \rightarrow N_2O + H_2O + N_2$$

Thus, the expected slope would be 0.5 and the intercept would be between the isotopic values of the nitrite and the azide, minus any isotopic fractionation.

## RESULTS AND DISCUSSION

**Overall Reaction Rate and Yield.** The overall reaction rate was highly dependent on pH and decreased exponentially as the pH of the reaction media was increased. The reaction rate did not appear to change significantly with nitrite concentration, contradicting the results observed by Stedman.[14] This was most likely due to the much lower concentrations used in this study (0.5−40 $\mu$M) compared with those used by Stedman (2−25 mM). Yields were quantitative, and there was no observed negative effect of allowing the samples to incubate hours beyond the required reaction time before quenching with the sodium hydroxide solution. Natural waters rich in organic matter had no adverse effect on blanks.

**Nitrous Oxide Separation.** The resulting aqueous product was very pure, and the cold trap between the low-flow liquid nitrogen trap and the GC column was thawed monthly. The material trapped in the cold trap appeared to consist only of trace amounts of water. The Carbosorb trap removed almost all carbon dioxide, which is isobaric with nitrous oxide. The trace amount of carbon dioxide was sufficiently separated from nitrous oxide on the 30-m GC column (Figure 1). Aside from the occasional change of the Carbosorb and magnesium perchlorate traps, nothing else had to be replaced due to contamination or clogging over a period of half a year.

Most of the dissolved oxygen sparged from the sample was not trapped by liquid nitrogen, but a trace amount remained. Oxygen needed to be completely separated from $N_2O$ because it can form $NO_2$ from ion−ion reactions with nitrogen in the mass spectrometer source. $NO_2$ is isobaric with $N_2{}^{18}O$, so even small amounts produce large errors. The 80-s pause between switching back to the low flow and releasing the sample onto the GC column was enough time to allow any trace amounts of oxygen to bleed off the final liquid nitrogen trap.

**Nitrogen Isotopic Fractionation.** The $\delta^{15}N$ values of nitrite and nitrate laboratory standards were determined on a continuous flow combustion IRMS system in our laboratory (Europa elemental analyzer connected to another Finnigan Mat 251). Aliquots of concentrated stock solutions were dried onto glass fiber filters and pressed in tin cups before combustion on the elemental analyzer. The $\delta^{15}N$ of the laboratory nitrous oxide standard was determined using the same system by injection through a port prior to the combustion furnace of the elemental analyzer. Standards were chosen to reflect a natural abundance range. A comparison of $\delta^{15}N$ of nitrite standards run using the combustion technique and the current technique is shown in Figure 2. Triplicate 50-mL nitrite samples with concentrations down to 0.5 $\mu$M had standard deviations of 0.1‰ or better using the new technique. The standard deviations of triplicate 50-mL nitrate samples with concentrations down to 0.5 $\mu$M were normally below 0.2‰.

The slope was close to the theoretical value of 0.5 as predicted from the 1:1 combination of nitrite-N and azide-N. The small deviation from the theoretical slope is likely due to a small but consistent blank effect, which was consistently between 2 and 3 nmol for a 50-mL sample size. Nitrite standards of 100 nmol/50 mL were used to make the plot in Figure 2, of which the blank would be on average 2.5% of the standard size. We could argue that the deviation from a slope of exactly 0.5 could be due to the blank:

$$0.500 - (0.025 \times 0.5) = 0.488$$

which is closer to the actual value of the experimentally determined slope of 0.48. Also consistent with the dependence of this slope on a nitrogen blank is the observation that the slope decreased proportionally for standard isotope series of lower concentrations.

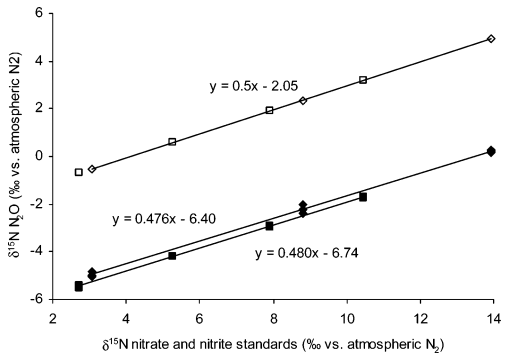


**Figure 2.** Nitrogen isotopic composition of nitrate standards converted to nitrous oxide using cadmium reduction followed by azide reduction (filled diamonds) and nitrite standards converted to nitrous oxide using azide (filled squares). Empty diamonds and squares represent the expected nitrogen isotopic values in the absence of fractionation of nitrate and nitrite, respectively. All samples were 50 mL of 2 $\mu$M nitrate or nitrite in seawater.

A more negative than expected isotopic intercept was obtained after the reaction between azide ion and nitrite for the plot of $\delta^{15}N$ nitrite versus $\delta^{15}N$ $N_2O$ (Figure 2). The intercepts for both nitrate and nitrite were equivalent, indicating the source of nitrogen isotopic fractionation to be a result of azide reduction of nitrite to nitrous oxide, not cadmium reduction of nitrate to nitrite. The expected intercept of −2.05‰ is based on the measured value of −4.1‰ for sodium azide. The difference between the measured and actual intercept is 4.4‰, indicating a fractionation factor of 8.8‰ due to the reaction between azide ion and nitrite. Since the yield of nitrous oxide showed quantitative conversion of nitrite, no isotropic fractionation with respect would be observed. This fractionation was very consistent within sample batches and fairly consistent (within 0.4‰) over a period of months. Fractionation was not sensitive to concentration (up to 40 $\mu$M) or temperature (15−30 °C).

**Oxygen Isotopic Fractionation and Exchange.** Nitrate standards of known $\delta^{15}N$, $\delta^{17}O$, and $\delta^{18}O$ (USGS34, $\delta^{15}N = -1.8$[air], $\delta^{18}O = -27.9$[vsmow] and USGS35, $\delta^{15}N = 2.7$[air], $\delta^{18}O = 57.5$[vsmow]) were analyzed to calibrate the method and determine the $\delta^{18}O$ of the final product, $N_2O$.[17] One available nitrite standard of known $\delta^{15}N$ and $\delta^{18}O$ was used (RSIL-N23, $\delta^{15}N = 3.7$[air], $\delta^{18}O = 11.4$[vsmow])[18] for standardization. At the time this paper was written, we did not have the resources to measure the $\delta^{18}O$ of the standard $N_2O$ gas. For this reason, the fractionation factor of oxygen for the reaction between azide and nitrite could not be determined. A plot of nitrous oxide derived from known

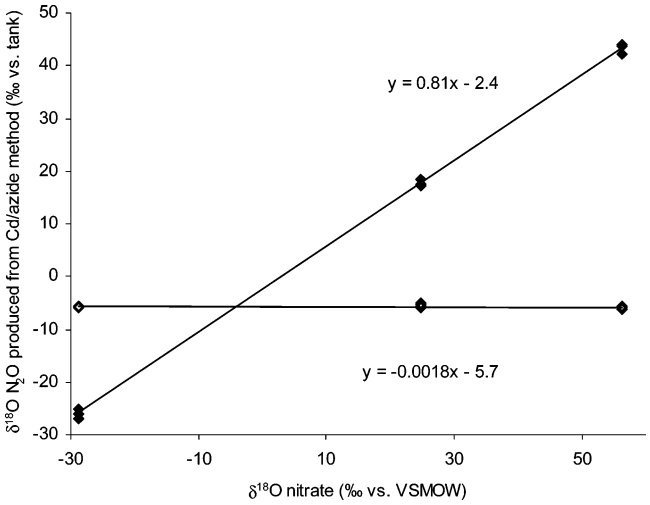


**Figure 3.** Oxygen isotopic composition of nitrate standards converted to nitrous oxide using cadmium reduction followed by azide reduction. Filled diamonds represent solutions buffered at pH 4.5 using acetic acid and sodium azide. Empty diamonds represent solutions in which hydrochloric acid was added prior to sodium azide for a resulting pH of 1.5. When hydrochloric acid is added to the solution before azide, complete oxygen exchange between nitrite and water was observed.

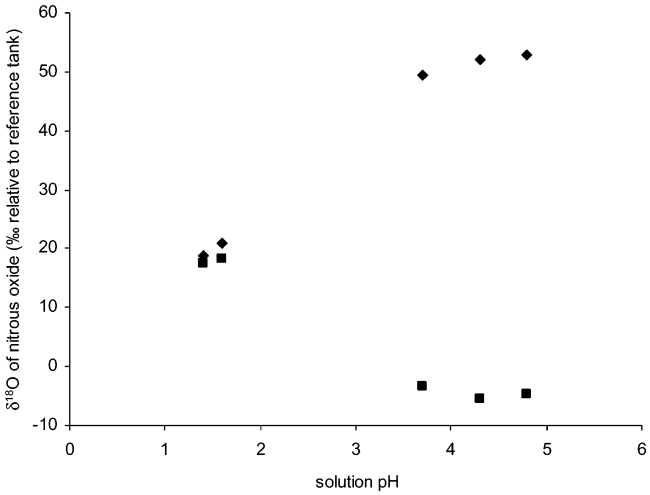


**Figure 4.** Oxygen isotopic composition of nitrate standards converted to nitrous oxide using cadmium reduction followed by azide reduction at various pH using hydrochloric acid, acetic acid, and sodium azide buffers. Diamonds represent nitrate with $\delta^{18}O = 56.3‰$, and squares represent nitrate with $\delta^{18}O = -28.7‰$.

$\delta^{18}O$ nitrate standards versus the published $\delta^{18}O$ values is shown in Figure 3. Since the $\delta^{18}O$ of the water was the same for all samples, deviations from a slope of 1 measure the degree of oxygen isotope exchange during the reaction. A slope near 0.81 is obtained and indicates an oxygen isotope exchange of 19% between water and nitrite. Little variation in this slope is observed between sample batches if reaction conditions are kept the same. There is no influence from varying nitrate or nitrite concentration. The fractionation offset for nitrate and nitrite $\delta^{18}O$ is the same for nitrate and nitrite samples, indicating the source of oxygen isotopic fractionation, and exchange is due to nitrite reduction to nitrous oxide and not cadmium reduction of nitrate.

**Ideal Experimental Conditions for Minimizing Oxygen Exchange.** The amount of oxygen exchange of the produced nitrous oxide is dependent on the competition between reactions 1−5 discussed in the introduction. The ideal experimental conditions were sought by varying the conditions that most affect these reactions, notably pH and halide concentration.

**Influence of pH.** To minimize oxygen exchange, we needed to create conditions that promote reaction 1 and inhibit reaction 4. Following Stedman's results, we could predict that a more basic reaction medium would result in less oxygen exchange. However, increasing pH was also expected to lower overall reaction rates. Fortunately, our results show a region of stability in the amount of oxygen exchange above pH 4 (Figure 4). However, our results also show that above pH 5 (close to the $pK_a$ of $HNO_2$), the reaction is very slow (or incomplete) and yields of $N_2O$ are very small (roughly 20% in 1 h). Several time series were done for 2 $\mu$M nitrite at pH 1.5, 4.0, 4.5, and 5.0, and the reactions were complete in 0.1, 3, 5, and 120 min, respectively. Therefore, for a practical analysis time and minimal oxygen exchange, the medium must be kept somewhere between pH 4 and 5.

These results are easily explained if we consider the relationship of protonated species/ionic species fraction versus pH of the reaction media. As pH increases, the concentration of the more reactive azide ion increases relative to hydrazoic acid. At the same time, the abundance of the reactive species $H_2NO_2^+$ decreases as pH increases. From our results, we can deduce that, above pH 5, $H_2NO_2^+$ is not at a sufficient concentration for the reaction to occur in a reasonable amount of time. We can also conclude that reaction 1 dominates and exchange with water becomes constant with small variations in pH above a pH of 4. It is probably not a coincidence that this is the pH where the ratio of $HNO_2/NO_2^-$ becomes less than the ratio of $N_3^-/HN_3$ (with increasing pH). Thus, from both theoretical and empirical tests, pH 4.5 is optimal for the conversion of nitrite to nitrous oxide using sodium azide.

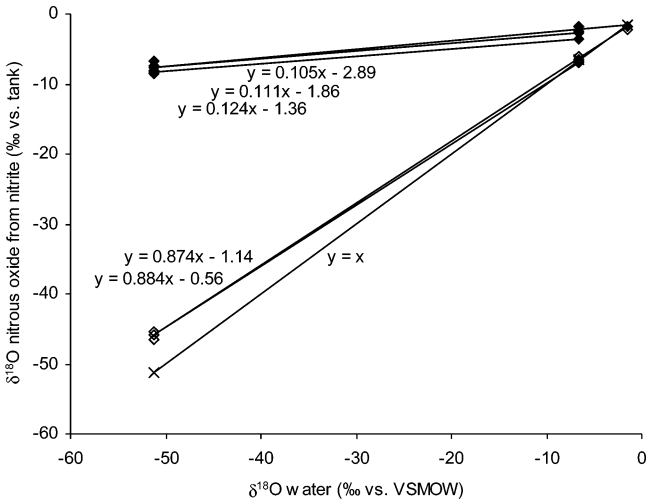


**Figure 5.** Repeated experiments using a single nitrite standard reduced to nitrous oxide in several different waters of known $\delta^{18}O$ at pH 4.5 (filled diamonds) and pH 1.5 (empty diamonds). The $Y = X$ plot represents the expected $\delta^{18}O$ value if the oxygen exchange between nitrite and water were 100%.

Water varying in $\delta^{18}O$ was used to quantify the oxygen exchange of nitrite during the azide reaction at pH 4.5 and 1.5. The results are shown in Figure 5. There was an exchange between 11 and 13% of the oxygen isotopes at pH 4.5 and ~88% exchange at pH 1.5. When hydrochloric acid is added to a nitrite solution several minutes prior to azide addition, 100% exchange

occurs between nitrite and water. An investigation into the use of this reaction for oxygen isotopic analysis of water is currently underway, and the results will be published in a separate paper.

It was difficult to target and consistently obtain a sample pH of 4.5 using sodium azide buffered with hydrochloric acid, so a search for a compatible buffer was performed. The best buffers for the target pH and compatibility with the reaction was found to be a combination of acetic acid and sodium acetate. For azide combined with the acetic acid/sodium acetate buffer, small variations in reagent addition or starting sample pH have little effect on the resulting pH and produced more consistent results as compared to sodium azide buffered with hydrochloric acid.

**Influence of Halides.** A comparison of nitrite $\delta^{18}O$ recovery from seawater, 0.6 M sodium chloride, 0.6 M sodium bromide, and DIW was performed. There was no significant exchange difference between the sea, chloride, and bromide waters, but the halide-free DIW was less consistent and resulted in greater exchange between nitrite and water. We can conclude that the rate of reaction 3 is greater than that of reaction 4. Therefore, the addition of chloride to freshwater samples is recommended to increase the rate of nitrous oxide formation relative to oxygen exchange. A chloride ion concentration higher than that of seawater (roughly 0.6 M) showed no additional benefit for lowering oxygen exchange and resulted in a decreased yield in nitrous oxide as solutions approached the saturation point of sodium chloride. There was no significant difference between sodium chloride and potassium bromide solutions.

## CONCLUSION

We have described a relatively simple natural abundance nitrogen and oxygen isotopic method utilizing a two-step chemical conversion to nitrous oxide followed by automated purge/trap IRMS analysis. It works well for seawater as well as freshwater down to low enviromental concentrations (0.5 $\mu$M). Our experience has shown it to be reliable and capable of high throughput. It has similar advantages to the dentrifier method (1) in producing nitrous oxide as the analyte for mass spectrometric analysis, a very low blank (<3 nmol) and very low sample mass reqiurement (<30 nmol). It has the further advantages of requiring off-the-shelf reagents and supplies, no need for maintenance of bacterial cultures, and applicability to samples incapable of sustaining bacterial activity. It is unique in that the nitrogen and oxygen isotopic abundance of nitrite is reliably determined in the presence of nitrate. The method's main disadvantage involves the safety precautions required for the handling of the azide reagent and its subsequent disposal. This technique has the further potential for analysis in samples below 0.5 $\mu$M through greater effort in blank reduction/correction and increased IRMS sensitivity. In the experiments comprising this study, the lower limit of the IRMS used was approached, not the lower limit of the method. We anticipate that the simplicity and precision of this method will be useful and attractive to many in pursuit of natural abundance isotopic data.

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## SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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