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Combining Denitrifying Bacteria and Laser Spectroscopy for Isotopic Analyses ($\delta^{15}N$, $\delta^{18}O$) of Dissolved Nitrate

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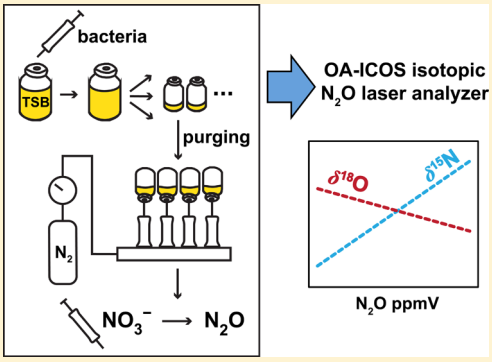
ABSTRACT: We present a novel approach for nitrogen ($\delta^{15}N$) and oxygen ($\delta^{18}O$) isotopic analysis of nitrate in water based on the isotopic analysis of N_2O produced from the conversion of NO_3^- by cultured denitrifying bacteria and off-axis integrated cavity output spectroscopy (OA-ICOS). The headspace N_2O was manually injected into an OA-ICOS isotopic N_2O laser analyzer through a syringe septum port. Sample analysis time was ~300 s. The use of OA-ICOS technology yields accurate and precise $\delta^{15}N$ and $\delta^{18}O$ results for dissolved nitrate samples when nonlinearity issues are considered. This new isotope analytical technique thus improves the isotopic analysis of nitrates by (i) providing accurate measurements of $\delta^{15}N$ and $\delta^{18}O$ without preconcentration, (ii) eliminating interferences by other gas substances (i.e., H_2O and CO_2), and (iii) reducing extensive maintenance and costs of isotope ratio mass spectrometers (IRMS). This approach will greatly streamline the identification and quantification of nitrate sources in aquatic systems.



Nitrate is an anionic N species that is considered one of the most common water contaminants associated with human health concerns.[1] Environmental issues associated with high levels of nitrate loadings include eutrophication and water quality degradation. The major sources of nitrates in water are runoff from fertilizers, septic tanks, and sewage and erosion of natural nitrate salt deposits. Anthropogenic nitrate inputs into the global nitrogen cycle have increased considerably and are negatively impacting aquatic ecosystems and human health.[1]

Nitrate sources in water and denitrification processes can be identified using nitrogen (^{15}N) and oxygen (^{18}O) stable isotopic compositions of nitrate.[2−4] Several methods have been developed to measure the $\delta^{15}N$ and $\delta^{18}O$ values of nitrate in freshwater and seawater: (i) the ion-exchange method that produces $AgNO_3$ salts,[5] (ii) the acetone method that produces anhydrous barium nitrate,[6] (iii) the azide method,[7] and (iv) the bacterial denitrification method[8,9] that reduces the NO_3^- to N_2O, which are ultimately analyzed for isotopic analysis using isotope-ratio mass spectrometry (IRMS). For the ion-exchange and acetone methods, samples of nitrate salts are generally combusted in an elemental analyzer (EA) to generate N_2 gas, which is introduced into an IRMS for $\delta^{15}N$ analysis; $\delta^{18}O$ measurements are conducted using the CO gas resulting from a high-temperature conversion system. For the bacterial denitrification and the azide method, analysis of ^{15}N and ^{18}O of N_2O can be performed via off-line cryoconcentration or online autosampler injection into an IRMS. A summary of the advantages and disadvantages of these methods was described previously.[10] Briefly, the ion-exchange and acetone method require a large sample size (target of ~20−200 μmol of NO_3^-) and the removal of interfering compounds, such as DOC, during sample preparation.[6,10] The bacterial denitrification and azide method can analyze low-concentration samples (e.g., 0.5 μmol L^{-1} of NO_3^-) and require smaller sample volumes (3 orders of magnitude smaller than the other two methods; e.g. 10 nmol of NO_3^-). In addition, these two methods are relatively inexpensive and less labor-intensive; however, they have other issues, such as the toxicity of some substances used in the azide method with the corresponding environmental and health protection procedures and a longer time for bacterial growth in the bacterial method.

All methods require a preliminary sample preparation step to convert the nitrate from water to obtain an appropriate substance to be analyzed (e.g., N_2O via headspace analysis, or nitrate salt via high-temperature conversion). In addition, they involve considerable cost and maintenance for accurate and precise analysis using IRMS techniques. For instance, IRMS methods require the elimination of interferences from other gases, such as H_2O and CO_2, by the installation of traps during the analysis of N_2O. Another consideration for accurate $\delta^{15}N$ determinations using N_2O is the possible bias in $\delta^{15}N$ values (~1−2 ‰) in cases where samples contain mass-independent ^{17}O variations due to relevant contributions of atmospheric nitrate in the sample.[11] Atmospheric nitrate is enriched in ^{17}O, and the routine correction in most IRMS data software that follows the mass-dependent relationship could overestimate the ratios of m/z 45 and m/z 44 used to calculate $\delta^{15}N$ values.

Laser spectroscopy methods have reduced some complexities and issues related to IRMS techniques for the analysis of stable isotopic composition of environmental samples.[12,13] Recently,

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laser gas analyzers based on off-axis integrated cavity output spectroscopy (OA-ICOS) have reported data comparable to that from IRMS approaches for many applications. Our aim was to develop a simple and inexpensive method for the analysis of nitrate for both ^{15}N and ^{18}O that is accurate, precise, and efficient for the application of environmental water monitoring programs. The method is based on the rapid and easy measurement of nitrate isotopic compositions by new laser N_2O analyzers, which allows the analysis of 20 samples in ~4−5 h without the need for considering mass-independent variations in sample ^{17}O, interference of other substances in the headspace, and time-consuming IRMS methods. We used the bacterial denitrification method for its simplicity, lower cost and labor intensity, its ability to analyze low NO_3^-concentration samples, and for its minor chemical and biological hazard in the laboratory safety issues. The precision and accuracy of our method were investigated using two potassium nitrate standards: one with a known isotopic composition of nitrogen and oxygen (the reference material IAEA-NO3), and one in-house standard that was developed by the Isotope Science Laboratory — University of Calgary (ISL-CSM23). In addition, we compared the results of our method with the isotopic composition obtained by IRMS. Our method was applied to natural water samples from rivers, and their isotope data are reported in this study to demonstrate the use of the method in environmental monitoring.

MATERIALS AND METHODS

Bacterial Culture Preparation. Nitrate sample preparation by the bacterial method has been reported previously.[8,9,14] In this study, cultures of denitrifying bacteria (*Pseudomonas aureofaciens*, ATCC no. 13985) were prepared for the conversion of NO_3^- to N_2O because these bacteria lack nitrous oxide reductase activity, which reduces N_2O to N_2. The working cultures were grown in 100 mL batches of tryptic soy broth (TSB) amended with 10 mM KNO_3, 7.5 mM NH_4Cl, and 36 mM KH_2PO_4,[9] which were inoculated with 0.8 mL of a starter culture (originally prepared in the Isotope Science Laboratory — University of Calgary). Before inoculation, liquid medium was prepared in serum bottles, which were crimp-sealed with 20 mm gray butyl septa (Wheaton, Millville, NJ) and then autoclaved the same day. Cultures were left to grow for 6−10 days to acquire enough bacteria and a complete utilization of nitrate amended for growth.

Sample Preparation. Culture cells from serum bottles were concentrated using centrifugation (6-fold, 3000 rpm for 45 min), and the cells after discarding the supernatant medium were then resuspended in fresh nitrate-free medium with a vortex shaker for ~30 s. The use of fresh nitrate-free medium results in lower blanks compared with using the spent medium in which the bacteria were grown.[14] This enhancement of the method was found to be independent of a variable cell concentration (3.7- to 10-fold). We dispensed 2.5 mL of concentrated cell culture into each 20 mL headspace sample vial. Vials were then sealed with Al-capped 20 mm gray butyl septa (Wheaton, Millville, NJ). Gray butyl septa were used following the recommendation from McIlvin and Casciotti,[14] whose study demonstrated negligible or little loss of N_2O and unchanged isotopic composition over several months when using this type of vial seals. Sealed vials were purged for 3 h with high-purity N_2 upside down in a purging manifold system with 25-gauge bubbling needles along with an inserted 25-gauge vent needle to produce an anaerobic environment and to remove any resulting N_2O from the previous processes. After purging, the vent needle was withdrawn, immediately followed by the bubbling needle, and the sample vial was labeled. The concentration of N_2O in the blanks for our method was 2 orders of magnitude lower than the laboratory standard at the regular target amount. Water samples and laboratory standards with sufficient amounts of dissolved nitrate (we targeted 175 nmol of NO_3^-) were injected into the vials with an airtight syringe and a 25-gauge needle. An incubation with the vial upside down was conducted overnight before isotopic analyses. By denitrification pathways, the theoretical amount of N_2O produced from a complete conversion of NO_3^- yields half the numbers of moles of N_2O ($2NO_3^- \rightarrow N_2O$). Specifically, the target amount of 175 nmol of NO_3^- produced a concentration of ~10 ppmV of N_2O in the instrument cavity. All materials used, such as the gray butyl septa, headspace sample vial, and aluminum seals, were prewashed and autoclaved.

Nitrate isotope standards (USGS34, USGS35, IAEA-NO3, and ISL-CSM23), which are KNO_3 except for USGS35 ($NaNO_3$), were dissolved in deionized distilled water to produce a concentration of 350 μmol/L. Riverine samples were collected in 125 mL HDPE bottles and were previously field-filtered to 0.45 μm pore size to eliminate bacteria that can affect the isotopic composition of the sample. Bottles containing prefiltered water samples were stored in a cooler in the field and frozen upon arrival at the lab. Nitrate concentrations from field samples (typically 0.5−10 mg L^{-1} of nitrate−NO_3^-) were analyzed at the National Laboratory for Environmental Testing (NLET), Environment Canada, Saskatoon. Concentrations were determined using automated colorimetric determination of nitrate−nitrite in water that uses a procedure in which nitrate is reduced to nitrite using an online cadmium reductor cell. The resulting nitrites react under acidic conditions with sulphanilamide to form a diazo compound that then couples with *N*-1(naphthyl)-ethylenediamine dihydrochloride to form a reddish purple azo dye. The absorption of the monochromatic radiation by the azo dye is proportional to the nitrate−nitrite concentration and is measured spectrophotometrically at 550 nm.

The amount of water of unknown samples required for isotope analysis depended on the nitrate concentration and therefore required similar amounts of nitrate in the 20 mL vials relative to the laboratory isotope standards. We preconcentrated all riverine samples to be comparable to the standards by freeze-drying the original collected sample, and the resulting solids were dissolved in deionized distilled water. Thus, we obtained an appropriate nitrate concentration in the sample to be analyzed for stable isotopes. A 2-fold preconcentration was performed for most of these samples (n = 24), but some (n = 7) were concentrated 4-fold, and one was not concentrated (1×). Rehydrated samples as well as the dissolved nitrate isotope standards were stored at 4 °C to avoid bacterial activity.

Isotopic Analysis. For the measurements of $\delta^{15}N$ and $\delta^{18}O$, the samples of headspace N_2O gas were analyzed using an OA-ICOS isotopic N_2O laser analyzer (Los Gatos Research, Mountain View, CA, USA; model 914-0027). According to the manufacturer, the measurement and operational concentration range of N_2O are 0−100 and 0−1000 ppmV, respectively. To fit within those ranges, a constant volume of 10 mL of headspace gas in the bacterial reaction vial was extracted off-line into a 100 mL gastight syringe (Hamilton, Reno, NV, USA; model 1100) coupled to a 22-gauge metal hub needle and manually injected into the analyzer through a

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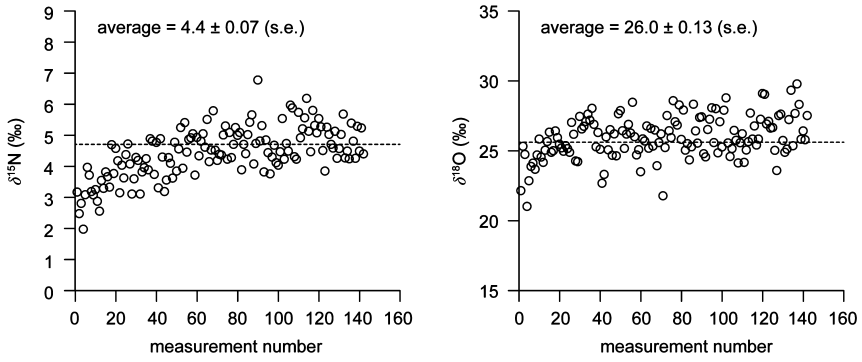

Figure 1. Typical measurements of $\delta^{15}N$ and $\delta^{18}O$ values for an injection analyzed with the isotopic N_2O analyzer. The N_2O gas sample was converted from a dissolved potassium nitrate reference material (IAEA-NO3), and the dashed line in the plot depicts the reference isotope values relative to AIR (+4.7 ‰ for $\delta^{15}N$) and VSMOW (+25.6 ‰ for $\delta^{18}O$).

syringe septum port mounted on the instrument. Before injecting the sample into the syringe port, the instrument pumped out the measurement ICOS cell and the internal sample tank and flushed twice with N_2O-free air (zero-air) via an inlet port (1/4 in. Swagelok) at the back panel configuration of the instrument at 5−10 psig. The instrument then pumped down to ∼5 Torr to be ready for sample injection, and the vacuum drew the gas from the syringe to the internal sample tank of the instrument, which retains the gas sample until the sample is fully injected. After injection, the gas sample was admitted and equilibrated in the measurement cell with an aliquot of N_2O-free air to reach ∼46 Torr. The sample was measured at a sampling rate averaging of 0.5 Hz for 300 s (∼142 measurements per sample over time in the measurement cell).

The instrument was warmed for at least 24 h before discrete sample injections to reach a relatively constant temperature in the cavity (∼41.5 °C) and was conditioned with 4−6 injections of N_2O gas at a constant concentration. A typical measurement of $\delta^{15}N$ and $\delta^{18}O$ values in the laser analyzer is shown in Figure 1. We gained accuracy integrating many measurements from the same sample gas introduced in the cavity from a headspace vial measuring at a sampling rate of 0.5 Hz for 300 s, and thus, we reported the mean value for each individual sample that integrates ∼142 measurements. The precision for each individual analysis (sample) can be relatively large, in addition to the occurrence of nonlinear trends, as shown in Figure 1. All this can be modified at a different sampling rate averaging and measurement time, but we did not test these variables in our study.

The isotopic laser analyzer measures the total concentration of the N_2O isotopologues ($^{14}N^{14}N^{16}O$, $^{14}N^{15}N^{16}O$, $^{15}N^{14}N^{16}O$, $^{14}N^{14}N^{18}O$) in the sample. The instrument does not measure any isotopologue with ^{17}O, which eliminates any potential overestimation of ^{15}N in samples containing mass-independent ^{17}O variations. Therefore, the absolute isotopic ratios were converted to the δ notation relative to AIR ($\delta^{15}N$) and VSMOW ($\delta^{18}O$) using at least two previously calibrated working standards or reference materials of known isotopic composition that were included in each batch for data normalization. All water samples and nitrate isotope standards were normalized to the known isotopic composition of the reference materials USGS34 (−1.8 ‰ for $\delta^{15}N$ and −27.9 ‰ for $\delta^{18}O$) and USGS35 (+2.7 ‰ for $\delta^{15}N$ and +57.5 ‰ for $\delta^{18}O$). Unlike ion sources from mass spectrometers, the laser spectroscopy analyzers cannot be tuned for maximum linearity; therefore, instrument linearity should be tested and quantified (see below).

Residual sample memory effects from the gastight syringe were negligible. During gas handling and injection, there might be a residual N_2O amount incorporated from AIR (∼0.3 ppmV) in the needle volume. For our method, the potential atmospheric N_2O contamination from air is extremely low (<∼0.002%) if we consider a mass balance model for our target concentration (∼10 ppmV). However, N_2 gas from an inflatable and leak-tight gas sampling bag (e.g., Tedlar bags, Ziploc brand Double Guard freezer bags) may be used to rinse the syringe needle to avoid atmospheric N_2O contamination between samples and reduce isotopic uncertainties. Ziploc freezer bags filled with N_2 were used in our experiments, and no residual syringe effects were observed. Regarding the amount of headspace gas extracted, we briefly evaluated this matter to optimize the N_2O produced in the sample vial and measurement in the analyzer. A correlation between the amount of NO_3^- injected, or subsequently the N_2O concentration measured, and the isotopic ratios was found when using distinct water sample injection volume (nmol) in the headspace vial. Therefore, we maintained an extracted volume of 10 mL, and the influence of sample amount used during bacterial denitrification processing on the $\delta^{15}N$ and $\delta^{18}O$ values was tested by measuring the isotopic composition of the resulting N_2O from different volumes of injected water in the headspace vial. This test was performed on vials with and without N_2 overpressure after overnight incubation to determine whether potential backfilling with air into the syringe might modify the measured δ values. Determinations of $\delta^{15}N$ and $\delta^{18}O$ were conducted with sample volumes from 250 to 1000 μL of the 350 μM solution of USGS34 standard, and isotopic ratios were calculated using constant injection volumes (∼175 nmol of nitrate) for the normalization nitrate isotope standards (USGS34 and USGS35).

Measurement uncertainty (including linearity effects) could be related to both the actual instrumental measurement of isotopic composition in N_2O and the sample preparation procedures. Thus, we tested the analysis of different amounts (50−300 μL) of the same gas (100% N_2O, diluted at 2.5% v/v) injected with a gastight syringe into a 20 mL headspace vial, which was previously pumped down to ∼0.02 Torr, flushed, and overpressured with N_2 (to avoid potential backfilling with air into the syringe). We then extracted 10 mL from the vial and injected it into the isotopic N_2O laser analyzer using the same procedure as for the samples from the nitrates.

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Table 1. Accuracy and Precision for Measured $\delta^{15}N$ and $\delta^{18}O$ of Nitrate Standards (IAEA-NO3 and ISL-CSM23) Using IRMS (by combustion and high-temperature conversion techniques, respectively) and Laser Spectroscopy (OA-ICOS)[a]

	$\delta^{15}N$ (‰)				$\delta^{18}O$ (‰)			
standard	IRMS	*n*	OA-ICOS	*n*	IRMS	*n*	OA-ICOS	*n*
IAEA-NO3	4.7 ± 0.14	4	4.6 ± 0.18	11	25.8 ± 0.21	6	25.8 ± 0.64	11
ISL-CSM23	0.7 ± 0.11	5	0.8 ± 0.17	8	15.7 ± 0.30	5	14.9 ± 1.15	8

[a]Isotopic values are expressed in per mil relative to AIR and VSMOW and were normalized using the nitrate standards USGS32, USGS34, and USGS35 for IRMS and USGS34 and USGS35 for laser spectroscopy. Expected isotope values for IAEA-NO3 are +4.7 ± 0.2 ‰ for $\delta^{15}N$ and +25.6 ± 0.4 ‰ for $\delta^{18}O$, and for ISL-CSM23 are +0.5 ± 0.16 ‰ for $\delta^{15}N$ and +15.3 ± 0.18 ‰ for $\delta^{18}O$ (provided by Isotope Science Laboratory — University of Calgary). Note: *n* = number of single measurements.

For comparison, the $\delta^{15}N$ and $\delta^{18}O$ values of KNO_3 standards IAEA-NO3 and ISL-CSM23 were also measured using continuous-flow isotope ratio mass spectrometry (CF-IRMS). For $\delta^{15}N$ measurements, nitrate salts were combusted in a Vario Microcube (Elementar Analysensysteme, Hanau, Germany), which employed a chemical trap system as an alternative to a GC column, and resulting gases were analyzed using an IRMS (Micromass Isoprime, Manchester, UK). For $\delta^{18}O$ measurements, samples were converted to H_2, N_2, and CO gas using a Thermo Scientific High Temperature Conversion Elemental Analyzer (TC/EA) whose glassy carbon reactor was set to 1400 °C and the gas chromatography (GC) column at 90 °C. The derived CO sample pulse was then introduced to a Delta V Plus isotope-ratio mass spectrometer (Thermo Scientific, Bremen, Germany) by a ConFlo IV Universal Interface system. However, the derived N_2 sample pulse can react with residual oxygen in the ion source forming $^{14}N^{16}O^+$ (m/z-30), which interferes with the CO peak and with reliable measurements of $\delta^{18}O$; therefore, the pulse of N_2 was diverted and diluted with He.[15,16] Moreover, the N_2 sample pulse was well separated from the CO peak using a relatively long GC column (1.5 m). When using the IRMS methods, the isotope ratios and the consequent δ values were calculated relative to a reference pulse of high-purity research grade N_2 and CO gases, which were automatically introduced using the reference gas injection box and the dual-inlet bellows, respectively. Isotopic values were normalized using the nitrate standards USGS32, USGS34, and USGS35. Analytical precision for IRMS was better than ±0.2 ‰ for $\delta^{15}N$ and ±0.4 ‰ for $\delta^{18}O$. All isotope δ values are reported in per mil (‰) deviations from the international standard (AIR and VSMOW, respectively).

RESULTS AND DISCUSSION

Accuracy and Precision. Measured $\delta^{15}N$ values from the dissolved nitrate of the IAEA-NO3 (+4.6‰) using laser spectroscopy (OA-ICOS) were very close to the expected average values for this reference material and to those measured $\delta^{15}N$ values using IRMS techniques (+4.7‰, Table 1). Similarly, the $\delta^{15}N$ values for ISL-CSM23 measured by IRMS (+0.7‰) and OA-ICOS (+0.8‰) were in good agreement (Table 1). In relation to $\delta^{18}O$, the measured values were also in good agreement with the expected values for IAEA-NO3 and with those measured using IRMS for both standards assessed (Table 1). However, measurements of $\delta^{18}O$ using OA-ICOS were less accurate relative to the determinations of $\delta^{15}N$. This was the case especially for ISL-CSM23, which should be considered as a standard for QA/QC control. Values of $\delta^{15}N$ and $\delta^{18}O$ measured by OA-ICOS technology yielded precisions comparable to those for the IRMS methods. Reproducibility of repeated measurements using OA-ICOS techniques yielded values close to ±0.2‰ for $\delta^{15}N$ and values close to ±0.6‰ for $\delta^{18}O$ in the case of IAEA-NO3 (Table 1), which is similar to the uncertainties associated with nitrate isotopic reference materials.[17] In addition, isotopic measurements for repeated injections of the same N_2O gas source at ∼10 ppmV N_2O yielded uncertainties better than ±0.3‰ and ±0.6‰ for $\delta^{15}N$ and $\delta^{18}O$, respectively (n = 10).

For data normalization, at least two isotope standards with widely separated isotopic values must be included in each batch of samples for analysis. In addition, if these standards are in a salt state, they should be dissolved in deionized distilled water to an adequate NO_3^- concentration and stored cool. A principal difference between using IRMS and OA-ICOS techniques is that the isotopic measurements using the isotopic N_2O laser analyzer are based on measured absorption spectra by spectroscopy methods, and the use of a comparative reference gas of known isotopic composition is not needed, although the calibration of the instrument and the measurement of real-time N_2O samples (e.g., atmospheric nitrous oxide) might need a local reference gas for normalization.

Linearity Effects. The laser instrument showed a linearity effect when using a range of N_2O volumes from a diluted N_2O gas source (Table 2). We found a positive effect for $\delta^{15}N$ (average: +0.11‰ per ppmV) and for $\delta^{18}O$ (+0.37‰ per ppmV) that displayed more ^{15}N-enriched and ^{18}O-enriched isotope values with increasing sample N_2O concentration, and this influence could be instrument-specific. However, an amount-dependence on isotope values for this method seems to behave as a combination of the instrument nonlinearity and other mechanisms associated with the sample preparation procedures (Figure 2). When varying amounts of NO_3^- were analyzed by the method, a positive trend with a slope of +0.22 and a negative trend with a slope of −0.19 were found for $\delta^{15}N$ and $\delta^{18}O$, respectively. This indicates that other mechanisms apart of the instrument nonlinearity are confounding the results for nitrate isotopic analysis. Specifically, these factors seem to affect in the same direction (i.e., higher ppmV, more ^{15}N-

Table 2. Linearity Effects for Different Sample Concentrations of N_2O Gas Taken from the Same Gas Source (100% N_2O, diluted to 2.5% v/v) Analyzed in Three Different Analytical Runs and Dates (A−C)[a]

analysis	$\delta^{15}N$ (‰ per ppmV)	$\delta^{18}O$ (‰ per ppmV)	*n*	range (ppmV)
A	+0.11	+0.37	9	5−15
B	+0.11	+0.34	11	5−15
C	+0.10	+0.39	4	5−20

[a]Vials were over-pressurized with N_2 to avoid potential backfilling with air into the syringe.

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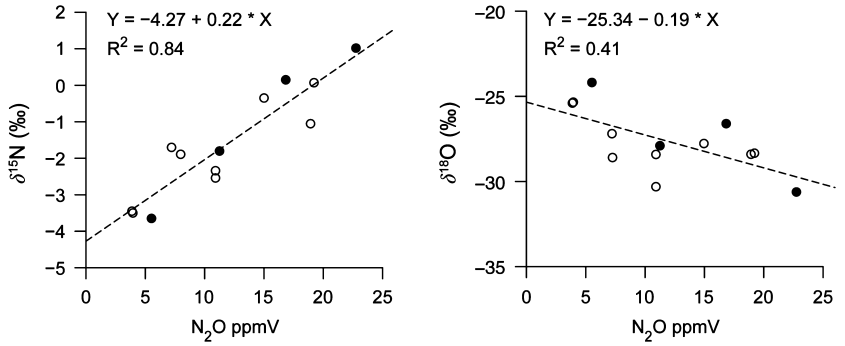

Figure 2. Influence of sample injection volume (as ppmV of N_2O measured in the cavity) on the $\delta^{15}N$ and $\delta^{18}O$ values for one laboratory standard (dissolved potassium nitrate reference material USGS34). Results were normalized to 175 nmol nitrate injections in cell culture vials using the standards USGS34 and USGS35. Empty circles depict those headspace vials that were overpressurized with N_2 before sample injection into the instrument, and solid circles, those that were not.

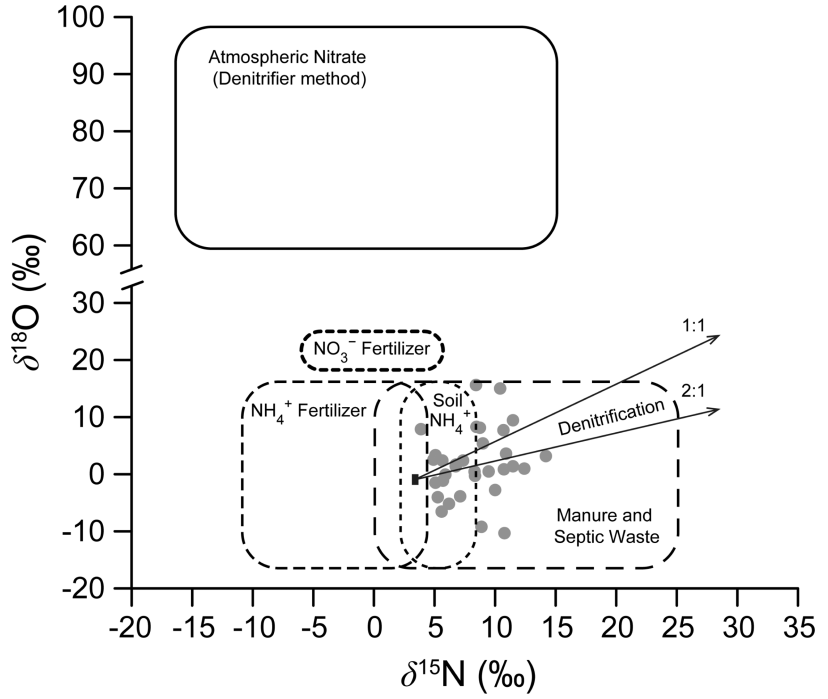

Figure 3. $\delta^{15}N$ and $\delta^{18}O$ values of riverine nitrate in the Lake Winnipeg watershed compared with the isotopic composition of nitrate from several N sources (adapted from ref 2).

enriched isotope values) for $\delta^{15}N$ and in the opposite direction for $\delta^{18}O$. We briefly investigated if overpressurizing headspace vials with N_2 before extracting the sample gas might change the effect, but this did not affect the trends (Figure 2). Thus, we speculate that a differential bacterial discrimination (plus instrument nonlinearity) depending on the nitrate amount added to the 20 mL closed system may be the reason for such an effect.

In summary, our results suggest that instrument nonlinearity seems to be relatively constant, but the sample amount dependency for our method needs further investigation to determine other mechanisms involved. Therefore, when natural water samples need to be measured for stable isotopes in nitrate, we propose two protocols for an accurate and precise analysis: (1) Analyze different volumes of injected water sample of a nitrate isotope standard to determine the linearity effect within the possible range of N_2O amount analyzed in the laser analyzer (Figure 2) and correct the isotopic ratios mathematically from this effect to a constant value of volume. (2) Inject relatively precise injection volumes of sample N_2O gas from nitrates into the cavity (e.g., 10 ppmV N_2O for our instrument). Hence, we suggest that this effect should be considered in every sequence run when using our method until a better understanding of the linearity mechanisms is known. Similar influences have been described for other OA-ICOS laser technologies, such as the liquid water analyzer;[12] however, in this case, those authors opted to recommend the second protocol (i.e., similar precise volumes of gas should be injected). If possible, we recommend following this recommendation because this would remove any uncertainty associated with the linearity effect correction.

Field Results. Preliminary $\delta^{15}N$ and $\delta^{18}O$ data from a large-scale research study in the Lake Winnipeg watershed (Canada) using our method is reported in Figure 3. These water samples were collected in May and July 2014, from the Assiniboine and Red Rivers, which ultimately drain into Lake Winnipeg at the south basin. The Assiniboine River connects with the Red River at the City of Winnipeg, and the Red River enters Canada

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through the U.S. border before that connection. Concentrations of NO_3^- for the riverine samples analyzed ranged from 0.8 to 12.5 mg L^{-1}. The smallest amount of NO_3^- injected into the sample vial, which was analyzed and corrected appropriately for nonlinearity issues, was ~85 nmol. Overall, $\delta^{15}N$ values ranged from +3.9‰ to +14.2‰, and $\delta^{18}O$ values ranged from −10.3‰ to +15.6‰. These values were variable and indicated a contribution of soil ammonium or manure/urban waste, or both, as the predominant sources of nitrate contamination in the watershed. The results of $\delta^{15}N$ and $\delta^{18}O$ data were in good agreement with the expected values for the Lake Winnipeg and the Red River watershed and with those previously analyzed by IRMS methods.[3]

CONCLUSIONS

The isotope analytical technique presented in this study using the bacterial denitrification method and OA-ICOS laser technology provided accurate and precise determinations of $\delta^{15}N$ and $\delta^{18}O$ values of nitrate, which are equivalent to IRMS methods. The use of the laser spectroscopy techniques eliminates (1) the preparative purification of headspace N_2O gas (e.g., cryo-concentration) before isotopic analysis, (2) the overestimation associated with variations in the ^{17}O content (e.g., atmospheric nitrate contribution), and (3) the need for a local reference gas during operation and reduces (4) maintenance and technical costs of mass spectrometers and peripherals, in addition to (5) their easier mobility and lower cost relative to IRMS systems. Moreover, the isotopic N_2O laser instruments, unlike IRMS, also have the ability to measure N_2O continuously in real time. This may open new research avenues in nitrogen cycling processes, such as sources and sinks of atmospheric N_2O. Dissolved nitrate from natural water samples can be processed rapidly by bacterial denitrification and analyzed by collecting the headspace to be then injected into the laser analyzer through a syringe septum port. Application of this novel technique will assist with the determination of relative contributions of nitrate sources, also potentially with the quantification of those contributions using (Bayesian) isotope-mixing models,[10] which will increase the knowledge of nitrogen cycling in aquatic ecosystems.

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Notes

The authors declare no competing financial interest.

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