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Nitrogen-15 and Oxygen-18 Natural Abundance of Potassium Chloride Extractable Soil Nitrate Using the Denitrifier Method

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In agroecosystems, most isotopic investigations of NO_3^- involve the use of tracers that are artificially enriched in ^{15}N . Although the dual isotope composition of NO_3^- — $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ is especially beneficial for understanding the origin and fate of NO_3^- , its use for KCl-extractable soil NO_3^- has been hampered by the lack of a suitable analytical technique. Our objective was to test whether the denitrifier method, whereby NO_3^- is reduced to N_2O before mass spectrometric analysis, can be used to determine the N and O isotopic composition of NO_3^- from 2 M KCl soil extracts. Several internationally accepted NO_3^- standards were dissolved in 2 M KCl, the conventional extractant for soil inorganic N, and inoculated with the bacterial strain *Pseudomonas aureofaciens* (ATCC no. 13985). The standard deviation of the NO_3^- standards analyzed did not exceed 0.2‰ for $\delta^{15}\text{N}$ and 0.3‰ for $\delta^{18}\text{O}$ values. After appropriate corrections, differences between our measured and consensus $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of standard NO_3^- generally were within the standard deviations given for the consensus values. Both $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values were reproducible among separate analytical runs. The method was also tested on genuine 2 M KCl extracts from unfertilized and fertilized soils. Depending on N fertilization, the soils had distinct $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values, which were attributed to amendment with NH_4NO_3 fertilizer. Hence, our data indicate that the denitrifier method provides a fast, reliable, precise, and accurate way of simultaneously analyzing the natural abundances of ^{15}N and ^{18}O in KCl-extractable soil NO_3^- .

The natural abundance of ^{15}N in NO_3^- is rarely used to investigate N dynamics in agroecosystem studies. Instead, most of these studies use ^{15}N -enriched substances. Furthermore, the O isotopic signature on soil NO_3^- has received even less attention, probably because suitable analytical techniques are lacking.

Kohl et al. (1971) were among the first to study whether N isotopes could be used to identify the origin of aqueous N in surface water. Their study sparked insightful discussions on the pros and cons of using natural ^{15}N abundance to study agricultural N dynamics. Taken alone, however, $\delta^{15}\text{N}$ values fail to distinguish among various NO_3^- sources, because the $\delta^{15}\text{N}$ values overlap and isotopic fractionation by various N transformations may further obscure the original source signature(s) (e.g., Kendall, 1998). When Amberger and Schmidt (1987) introduced the measurement of $\delta^{18}\text{O}$ values, however, a new technique became available to better differentiate between NO_3^- sources and understand NO_3^- transformations. The $\delta^{18}\text{O}$ values in conjunction with $\delta^{15}\text{N}$ values of NO_3^- have been used extensively and successfully in hydrological studies to address NO_3^- sources and transformations (e.g., Aravena et al., 1993; Wassenaar, 1995; Durka et al., 1994; Chang et al., 2002; Rock and Mayer, 2002, 2004), although rarely in soil-plant studies. Högberg (1997) noted that the combined use of $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values may provide a powerful tool to elucidate soil N sources and transformations.

Various techniques are available to determine the N isotopic composition of NO_3^- . Diffusion techniques (e.g., Sebilio et al., 2004) are the most common; however, they do not enable measurement of $\delta^{18}\text{O}$ values of NO_3^- . To measure the N isotopic composition of NO_3^- , Stevens and Laughlin (1994) used an abiotic conversion of NO_3^- to N_2O , but they focused on ^{15}N -enriched samples, and did not assess the O isotopic composition of NO_3^- . Currently, the most widely used method to determine the O isotopic composition of NO_3^- is based on extracting aqueous NO_3^- using an anion exchange resin, and subsequent purification as AgNO_3 (Silva et al., 2000). This method, however, is not directly applicable to highly saline solutions, such as 2 M KCl extracts from soils, as such solutions interfere with the ion exchange processes.

Recently, a new technique, the denitrifier method (Sigman et al., 2001; Casciotti et al., 2002), was developed to determine the N and O isotopic composition of NO_3^- in freshwater and seawater. The latter has total dissolved solids (TDS) of about 33 000 mg L⁻¹, whereas 2 M KCl soil extracts have TDS at least five times higher. Our aim was to test whether the denitrifier method can be used to determine the N and O isotopic composition of NO_3^- from 2 M KCl extracts.

MATERIALS AND METHODS

The denitrifier method (Sigman et al., 2001; Casciotti et al., 2002) is based on bacterial reduction of NO_3^- to N_2O via a bacterium that lacks nitrous oxide reductase, so further reduction to N_2 does not occur. This enables simultaneous determination of both $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of the sample NO_3^- by measuring $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of the produced N_2O . In contrast, the conventional ion exchange method may require separate mass spectrometric analyses of $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values in purified AgNO_3 . A detailed description of the denitrifier method for determining the $\delta^{15}\text{N}$

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value of NO_3^- can be found in Sigman et al. (2001), and for the $\delta^{18}\text{O}$ value of NO_3^- in Casciotti et al. (2002).

In brief, we used the bacterial strain *Pseudomonas aureofaciens* (ATCC no. 13985) to reduce NO_3^- to N_2O . A fresh culture was prepared for each sample set by inoculating a tryptic soy broth medium (BD Diagnostics, Sparks, MD) amended with KNO_3 , KH_2PO_4 , and NH_4Cl in 125-mL serum bottles. After 7 d of growth, the bacteria were harvested and 2.25-mL aliquots were distributed among 20-mL headspace vials for sample analysis. Before injecting the samples, sample incubation vials containing bacteria plus medium were crimp sealed with septa caps, flushed with N_2 , and an antifoaming agent (Antifoam B, R06436, EMD Chemicals, Gibbstown, NJ) was added. We compared two addition times for the antifoaming agent: one during medium preparation and one just before crimp sealing the sample incubation vials. This test was done because it was noted that certain samples for which the antifoam was added during medium preparation still produced quite a bit of foam during sample analysis. This, in turn, posed a technical challenge, as liquid may enter the capillaries used to extract the N_2O . After sample injection, the vials were inverted and incubated overnight at room temperature to achieve complete reduction of NO_3^- to N_2O . Knowing the sample NO_3^- concentrations, the injected sample volumes were adjusted such that 50 nmol of NO_3^- (producing 25 nmol of N_2O) would be analyzed for every sample. This is essential for using the correction procedure, which is discussed below, of the raw (idem measured) δ values. We were able to analyze smaller amounts (e.g., 10 nmol, data not shown), as previously demonstrated by Sigman et al. (2001). We chose 50 nmol, as this resulted in a clear and strong signal of about 6 V for Mass 44 on the isotope ratio mass spectrometer that we used. Analyses were performed in the Isotope Science Laboratory (ISL) at the University of Calgary, Calgary, AB, Canada.

The instrumentation used to analyze isotopes of N_2O produced in the incubated sample vials included the following in-line components (all from Thermo Electron Co., Bremen, Germany, except where indicated): a modified PreCon trace gas concentrator, an HP6890 gas chromatograph with an HP-PLOT U column (Agilent Technologies, Palo Alto CA), a GC/C-TC interface, and a Delta Plus XL mass spectrometer. In essence, continuous flow isotope ratio mass spectrometry was used to determine the $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of N_2O derived from reduction of NO_3^- . The PreCon was modified through the addition of a Nafion gas drier (Perma Pure LLC, Toms River, NJ) to ensure thorough dehumidification, two pieces of Bev-A-Line IV tubing to which stainless steel needles were attached that had different lengths, and a holder for 20-mL headspace vials in place of the 100-mL flasks normally used for the PreCon.

To test whether the denitrifier method (Sigman et al., 2001; Casciotti et al., 2002) can be used to determine the N and O isotopic composition of NO_3^- from 2 M KCl soil extracts, four international NO_3^- standards and one internal laboratory standard were dissolved in 2 M KCl. Some

of these standards were used as references to correct the isotope measurements, and others were treated as samples for an independent assessment of analytical performance. Blanks, which were composed of the solvent 2 M KCl, were also analyzed during each run. In addition to the NO_3^- standards and blank KCl, we also analyzed 2 M KCl extracts of surface soil samples (0–15 cm) from unfertilized and fertilized plots and the applied NH_4NO_3 fertilizer (prills crushed and dissolved in 2 M KCl) to further assess the method. The extraction protocol was as follows: 10 g of air-dry soil and 50 mL 2 M KCl were added to 125-mL polypropylene bottles; these were placed horizontally on a reciprocating shaker (Eberbach Corp., Ann Arbor, MI) for 1 h, after which the samples were filtered using Fisherbrand Q2 filter paper.

The soil samples had been collected shortly after fertilization in May 2006 from plots under two contrasting management regimes in a field experiment with three replicates. Thus, for this study, six plots under two contrasting management regimes were sampled. Two soil cores (each 3.7-cm diameter) were combined by depth for each plot. For this study, the 0- to 15-cm depth increment was air dried and crushed to pass a 2-mm sieve. The experimental plots are part of the irrigated cropping systems study designated Rotation U and located at Agriculture & Agri-Food Canada's Lethbridge Research Centre (AAFC-LRC). Nitrate-N and NH_4^+ -N concentrations in the soil extracts and in the dissolved fertilizer prills were determined colorimetrically via segmented continuous flow (AutoAnalyzer 3, Bran+Luebbe GmbH, Norderstedt, Germany) at AAFC-LRC.

The international standards used were IAEA- NO_3 , USGS-34, USGS-35, and USGS-32. The internal laboratory standard was ISL-K NO_3 . The standards were used to prepare solutions of 2 M KCl containing NO_3^- at a concentration ($[\text{NO}_3^-]$) of 3.07 mg L $^{-1}$ for each standard, except USGS-35 for which $[\text{NO}_3^-]$ was 3.65 mg L $^{-1}$. The given $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of the NO_3^- standards used are listed in Table 1, which is based in part on data obtained from IAEA (2004). The δ values are defined as follows:

$$\delta_{\text{sample}} (\text{‰}) = \left(\frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) 1000$$

where R is the $^{15}\text{N}/^{14}\text{N}$ or $^{18}\text{O}/^{16}\text{O}$ ratio of the sample or a standard. The $\delta^{15}\text{N}$ values are reported relative to air N_2 , and $\delta^{18}\text{O}$ values relative to Vienna Standard Mean Ocean Water.

During each analysis, the N_2O produced from the various samples was compared with laboratory N_2O reference gas introduced via the bellows of the dual inlet connected to the Delta Plus XL. The absolute $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of the laboratory N_2O reference gas are immaterial, because, as will be discussed below, some of the international standards were used as absolute references to correct the other analyses.

RESULTS AND DISCUSSION

First, results obtained from two distinct bacterial batches (bat1 and bat2) are presented for analysis of NO_3^- standards. Note that bat2 was subdivided into two sets (bat2a and bat2b) to compare the two addition times for the antifoaming agent. The agent was added during medium preparation for bat2a, and just before the vials were crimp sealed for bat2b. Comparisons among the three batches enabled assessment of the precision, accuracy, and reproducibility of the denitrifier method for $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of NO_3^- in 2 M KCl. Second, results obtained for analysis of 2 M KCl-extractable NO_3^- in fertilized and unfertilized soils and of the added fertilizer are discussed.

The continuous flow isotope ratio mass spectrometry system used to extract and analyze the N_2O produced from reduction

Table 1. Given $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of internationally accepted and laboratory NO_3^- standards.

Standard	$\delta^{15}\text{N}_{\text{nitrate}}$	$\delta^{18}\text{O}_{\text{nitrate}}$
	‰	
IAEA- NO_3 †	+4.7 ± 0.2	+25.6 ± 0.4
USGS-32†	+180 ± 1.0	+25.7 ± 0.4
USGS-34†	−1.8 ± 0.2	−27.9 ± 0.6
USGS-35†	+2.7 ± 0.2	+57.5 ± 0.6
ISL-K NO_3 ‡	+4.6	+19.1 ± 1.2

† $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values from IAEA (2004).

‡ Laboratory standard.

of sample NO_3^- by the bacterial strain *Pseudomonas aureofaciens* (ATCC no. 13985) provided excellent chromatographic resolution of the N_2O (data not shown). Samples were analyzed individually, and each analysis took about 17 min. Hence, with our current system using manual sample changing, 22 samples (including instrument checks) can be analyzed in a typical 8-h workday. Manual changing of samples contained in septum-capped 20-mL headspace vials involved inserting first a long inlet needle that was submerged in the liquid, and second a short outlet needle that remained in the headspace above the liquid (and foam). Integrating an autosampler would significantly increase sample throughput.

Precision

The precision of the denitrifier method was assessed with bat1. This batch included three blanks (2 M KCl), three samples of IAEA-NO3 and USGS-34, five samples of USGS-35 and ISL-KNO3, and six samples of USGS-32. Note that the raw (or measured) $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values given below have already been corrected for mass interference due to the contributions of ^{17}O to Masses 45 and 46 using the same ISODAT 2.0 software as used for instrument control, data capture, and signal processing.

Nitrogen-15 Natural Abundance Values

The raw $\delta^{15}\text{N}$ values were $4.5 \pm 0.1\text{‰}$ ($n = 3$) for IAEA-NO3, $-1.8 \pm 0.1\text{‰}$ ($n = 3$) for USGS-34, $3.8 \pm 0.1\text{‰}$ ($n = 5$) for USGS-35, $175.3 \pm 0.2\text{‰}$ ($n = 6$) for USGS-32, and $4.5 \pm 0.1\text{‰}$ ($n = 5$) for ISL-KNO3 (Table 2). For blank KCl, the mass 44 peak areas were very small (< 200 mV), and amounted to $\sim 2.3\%$ of the overall average mass 44 peak areas recorded for the NO_3^- standards (Table 2). The analytical precision for $\delta^{15}\text{N}$ values of NO_3^- in 2 M KCl determined by the denitrifier technique is excellent, as the standard deviation (σ) of the NO_3^- standards analyzed did not exceed 0.2‰.

Oxygen-18 Natural Abundance Values

The measured raw $\delta^{18}\text{O}$ values were $64.7 \pm 0.2\text{‰}$ ($n = 3$) for IAEA-NO3, $12.8 \pm 0.2\text{‰}$ ($n = 3$) for USGS-34, $96.4 \pm 0.1\text{‰}$ ($n = 5$) for USGS-35, $64.9 \pm 0.3\text{‰}$ ($n = 6$) for USGS-32, and $61.5 \pm 0.2\text{‰}$ ($n = 5$) for ISL-KNO3 (Table 2). Based on the results obtained, it can be concluded that the precision for $\delta^{18}\text{O}$ values of NO_3^- from 2 M KCl extracts determined by the denitrifier technique is excellent, as the σ of the samples analyzed did not exceed 0.3‰.

Accuracy

The accuracy of the denitrifier method was assessed with bat1 and bat2. The latter included the same sample set as bat1, except that only one sample of each standard was prepared with bat2a and bat2b. To obtain the "true" $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of the sample NO_3^- based on the raw (or measured) $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of the analyzed N_2O , different corrections need to be applied to the $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values.

Nitrogen-15 Natural Abundance Values

The only correction needed for the $\delta^{15}\text{N}$ values is a blank correction, as the conversion of NO_3^- to N_2O is complete after an overnight incubation (Sigman et al., 2001). The IAEA-NO3 was used as the absolute reference for $\delta^{15}\text{N}$ analysis. Hence, to correct the raw $\delta^{15}\text{N}$ values, first the $\delta^{15}\text{N}$ value of the blank was estimated based on the given and measured $\delta^{15}\text{N}$ values of IAEA-NO3, and measured areas of Mass 44 with the following equation:

$$\delta_{\text{blank}} = \frac{(\delta \text{ AM44})_{\text{IAEA-NO}_3\text{meas}}}{\text{AM44}_{\text{blank}}} \quad [1]$$

$$- \frac{\delta_{\text{IAEA-NO}_3\text{known}} (\text{AM44}_{\text{IAEA-NO}_3\text{meas}} - \text{AM44}_{\text{blank}})}{\text{AM44}_{\text{blank}}}$$

where AM44 = area of Mass 44.

Then to calculate the blank-corrected $\delta^{15}\text{N}$ value for a specific sample, the following equation was used:

$$\delta_{\text{sample}} = \frac{\delta_{\text{meas}} \text{AM44}_{\text{meas}} - \delta_{\text{blank}} \text{AM44}_{\text{blank}}}{\text{AM44}_{\text{meas}} - \text{AM44}_{\text{blank}}} \quad [2]$$

where meas = raw (or measured) values for the sample.

The corrected and given or consensus $\delta^{15}\text{N}$ values of USGS-34, USGS-32, and ISL-KNO3 were basically identical, except for USGS-35 (Table 3). The difference between the corrected and given $\delta^{15}\text{N}$ values ($\Delta\delta^{15}\text{N}$) of the three former standards was within the standard deviation of the given $\delta^{15}\text{N}$ values (Table 3). For both bat1 and bat2, $\Delta\delta^{15}\text{N}$ was $\pm 0.1\text{‰}$ for USGS-34 with a given σ of 0.2‰, -0.6 to -0.9‰ for USGS-32 with a given σ of 1.0‰, and 0.0 to 0.2‰ for ISL-KNO3. The only exception was USGS-35, for which the $\Delta\delta^{15}\text{N}$ was consistently between 1.1 to 1.3‰ relative to its given $\delta^{15}\text{N}$ value, which has a σ of 0.2‰. This difference between the given and corrected $\delta^{15}\text{N}$ values of USGS-35 was also observed when we used deionized water as a solvent (data not shown). The USGS-35 sample, an atmospherically derived NO_3^- salt, has a significant mass-independent ^{17}O anomaly (Böhlke et al., 2003). Sigman et al. (2001) noted that the $\delta^{15}\text{N}$ values of atmospheric NO_3^- may be overestimated by 1 to 2‰ with the denitrifier method if it is assumed that there is no mass-independent ^{17}O anomaly. This explains the discrepancy observed for the $\Delta\delta^{15}\text{N}$ of USGS-35 relative to the other NO_3^- standards, as our software assumed a mass-dependent relationship to correct Mass 45 for ^{17}O contribution. Coplen et al. (2004) discussed in detail the determination of $\delta^{15}\text{N}$ values of NO_3^- containing mass-independent ^{17}O , such as atmospherically derived NO_3^- . Otherwise, accuracies were consistently good and similar for

Table 2. Averages and corresponding standard deviations of amplitude of Mass 44, area of Mass 44, and raw $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of NO_3^- standards prepared with bacterial batch bat1.

Sample	Amplitude of Mass 44		Area of Mass 44		Raw $\delta^{15}\text{N}_{\text{nitrate}}^\dagger$		Raw $\delta^{18}\text{O}_{\text{nitrate}}^\dagger$		n
	Avg.	SD	Avg.	SD	Avg.	SD	Avg.	SD	
	— mV —		— Vs —		— ‰ —				
Blank‡	161	5	0.564	0.016	—	—	—	—	3
IAEA-NO3	5685	425	24.504	0.234	4.5	0.1	64.7	0.2	3
USGS-34	5766	324	24.251	0.070	-1.8	0.1	12.8	0.2	3
USGS-35	5603	222	23.649	0.585	3.8	0.1	96.4	0.1	5
USGS-32	6088	250	25.264	0.908	175.3	0.2	64.9	0.3	6
ISL-KNO3	5823	261	24.735	0.556	4.5	0.1	61.5	0.2	5

† Includes correction for mass interference due to the ^{17}O contribution to Masses 45 and 46.

‡ Prepared with 2 M KCl.

bat2a and bat2b: the denitrifier method provided accurate $\delta^{15}\text{N}$ values of NO_3^- derived from 2 M KCl extracts.

Oxygen-18 Natural Abundance Values

In addition to a blank correction like that applied to the $\delta^{15}\text{N}$ values, the raw $\delta^{18}\text{O}$ values require further corrections (Sigman et al., 2001; Casciotti et al., 2002). These are due to: (i) isotopic fractionation during reduction of NO_3^- to N_2O , as only one of every six O atoms within the sample NO_3^- is preserved within the produced N_2O , and (ii) exchange of O atoms between sample water and intermediate N compounds produced during NO_3^- reduction to N_2O (Sigman et al., 2001; Casciotti et al., 2002). The ATCC no. 13985 strain of *Pseudomonas aureofaciens* was selected to reduce NO_3^- to N_2O because it results in the lowest O exchange between the intermediate N compounds and sample water (Casciotti et al., 2002).

To correct the measured $\delta^{18}\text{O}$ values, two approaches may be taken, as described by Casciotti et al. (2002). One approach involves the use of one absolute reference, namely IAEA-NO3, whereas the other approach involves the use of two absolute references. In the former, "exchange" and "blank" are determined separately and involve the analysis of additional samples produced with several distinct ^{18}O -enriched waters. The latter, however, enables the determination of a correction factor including all the factors influencing the analysis of the $\delta^{18}\text{O}$ values. Although Casciotti et al. (2002) felt the second approach might be better, they used the first approach because two NO_3^- standards with well-characterized $\delta^{18}\text{O}$ values were not yet available. We opted to use the second approach, and chose IAEA-NO3 and USGS-34 as the two absolute references.

To calculate the correction factor the following equation was used, which was derived from an equation in Casciotti et al. (2002):

$$\text{correction factor} = \frac{(\delta^{18}\text{O}_{\text{IAEA-NO3}} - \delta^{18}\text{O}_{\text{USGS-34}})_{\text{known}}}{(\delta^{18}\text{O}_{\text{IAEA-NO3}} - \delta^{18}\text{O}_{\text{USGS-34}})_{\text{measured}}} \quad [3]$$

To use Eq. [3], one has to ensure that the same amount of N_2O is produced from each sample, as this was the assumption used when this equation was derived (Casciotti et al., 2002). Another point to note is that the correction factor varies among bacterial batches (observed both for NO_3^- dissolved in deionized water [data not shown] or 2 M KCl). The correction factors calculated for the three bacterial batches (idem analytical runs) used in this study, bat1, bat2a, and bat2b, were 1.030954, 1.004506, and 1.029916, respectively. Thus this correction requires that IAEA-NO3 and USGS-34 standards are included in every sample set or run. This concurs with Casciotti et al. (2002), who noted that each batch of bacteria behaves slightly differently.

To calculate the corrected $\delta^{18}\text{O}$ value for a specific sample, the following equation was used, which is based on Eq. [4] in Casciotti et al. (2002):

$$\delta^{18}\text{O}_{\text{sample}} = \delta^{18}\text{O}_{\text{IAEA-NO3,known}} + (\delta^{18}\text{O}_{\text{measured}} - \delta^{18}\text{O}_{\text{IAEA-NO3,measured}}) \text{correction factor} \quad [4]$$

The corrected and given $\delta^{18}\text{O}$ values of USGS-35, and USGS-32 were nearly identical, but those for ISL-KNO3 differed (Table 3). The difference between the corrected and given $\delta^{18}\text{O}$ values ($\Delta\delta^{18}\text{O}$) for the first two standards generally was within the σ of the given $\delta^{18}\text{O}$ values (Table 3). For both bat1 and bat2, $\Delta\delta^{18}\text{O}$ was -0.1 to 0.7 ‰ for USGS-35 with a given σ of 0.6 ‰, and -0.2 to 0.1 ‰ for USGS-32 with a given σ of 0.4 ‰. The only exception was ISL-KNO3, for which the $\Delta\delta^{18}\text{O}$ was consistently between 2.6 to 3.2 ‰ relative to the given $\delta^{18}\text{O}$ value with $\sigma = 1.2$ ‰. Note that this difference between the given and corrected $\delta^{18}\text{O}$ values of ISL-KNO3 was also observed when deionized water was used as the solvent (data not shown). As for the $\delta^{15}\text{N}$ values, no difference in the accuracy was observed between bat2a and bat2b. Hence, based on the results obtained, the denitrifier method provides accurate $\delta^{18}\text{O}$ values of NO_3^- derived from 2 M KCl extracts.

Table 3. Average corrected $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of NO_3^- standards prepared with bacterial batches bat1 and bat2. Also shown the differences between the corrected δ values and the known δ values for a specific standard.

Bacterial batch	Sample†	Average corrected $\delta^{15}\text{N}_{\text{nitrate}}^\ddagger$	Average corrected $\delta^{18}\text{O}_{\text{nitrate}}^\S$	n	$\Delta\delta^{15}\text{N}^\P$	$\Delta\delta^{18}\text{O}^\#$
		‰	‰		‰	
bat1	IAEA-NO3	4.7	25.6	3	na††	na††
	USGS-34	-1.7	-27.9	3	0.1	na††
	USGS-35	4.0	58.2	5	1.3	0.7
	USGS-32	179.4	25.8	6	-0.6	0.1
	ISL-KNO3	4.8	22.3	5	0.2	3.2
bat2a	IAEA-NO3	4.7	25.6	1	na††	na††
	USGS-34	-1.9	-27.9	1	-0.1	na††
	USGS-35	3.8	57.4	1	1.1	-0.1
	USGS-32	179.1	25.5	1	-0.9	-0.2
	ISL-KNO3	4.6	21.8	1	0.0	2.7
bat2b	IAEA-NO3	4.7	25.6	1	na††	na††
	USGS-34	-1.7	-27.9	1	0.1	na††
	USGS-35	4.0	58.0	1	1.3	0.5
	USGS-32	179.4	25.5	1	-0.6	-0.2
	ISL-KNO3	4.6	21.7	1	0.0	2.6

† Refer to Table 1 for given $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values.

‡ Corrected for blank contribution using IAEA-NO3 as absolute reference and calculating $\delta^{15}\text{N}$ value of blank based on given and measured $\delta^{15}\text{N}$ values of IAEA-NO3.

§ Corrected for blank contribution, O exchange with water, and O fractionation during conversion of NO_3^- to N_2O using IAEA-NO3 and USGS-34 as absolute reference.

¶ $\Delta\delta^{15}\text{N}$ = corrected $\delta^{15}\text{N}$ - given $\delta^{15}\text{N}$.

$\Delta\delta^{18}\text{O}$ = corrected $\delta^{18}\text{O}$ - given $\delta^{18}\text{O}$.

†† Not applicable as IAEA-NO3 was used to calibrate the $\delta^{15}\text{N}$ values.

†† Not applicable as IAEA-NO3 and USGS-34 were used to calibrate the $\delta^{18}\text{O}$ values.

Reproducibility

The reproducibility of the denitrifier method was assessed by comparing the corrected $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values obtained from bat1 and bat2. As can be seen from Table 3, both the $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values were reproducible between different runs for which distinct batches

of the bacterial strain *Pseudomonas aureofaciens* (ATCC no.13985) were used. Furthermore, adding the antifoam agent either during medium preparation or just before the vials were crimp sealed did not affect the results, as no difference was observed in the corrected δ values obtained from bat2a and bat2b.

Example: Comparison of Fertilized and Unfertilized Soils

After testing the denitrifier technique on NO_3^- standards (with given $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values) dissolved in 2 M KCl, we used 2 M KCl to extract NO_3^- from soil samples collected from the 0- to 15-cm layer under three replicate plots of two contrasting treatments: unfertilized and NH_4NO_3 fertilized (100 kg N ha^{-1}) silage corn (Table 4). In addition, three samples of blank 2 M KCl used for the extraction and three samples of the NH_4NO_3 fertilizer applied to the soil were analyzed. Included within the sample run were also the international standards IAEA-NO3 and USGS-34 to correct the raw δ values obtained, and USGS-35 to assess the accuracy–precision–reproducibility of the run. The international standards were dissolved in 2 M KCl, and then treated the same way as the soil extracts during sample preparation.

The average NO_3^- -N concentration was $6.9 \pm 0.1 \text{ mg L}^{-1}$ ($n = 3$) for the fertilizer, $8.2 \pm 1.5 \text{ mg L}^{-1}$ ($n = 3$) for the fertilized soil extracts, and $2.3 \pm 0.5 \text{ mg L}^{-1}$ ($n = 3$) for the unfertilized soil extracts (Table 4). The average NH_4^+ -N concentration was $8.4 \pm 0.1 \text{ mg L}^{-1}$ ($n = 3$) for the fertilizer, $2.8 \pm 1.0 \text{ mg L}^{-1}$ ($n = 3$) for the fertilized soil extracts, and $0.7 \pm 0.0 \text{ mg L}^{-1}$ ($n = 3$) for the unfertilized soil extracts (Table 4).

Based on the NO_3^- concentrations, the sample or extract volume added to the 20-mL headspace vials was adjusted to obtain 50 nmol of NO_3^- , as discussed above. In turn, the sample volume added ranged from 0.08 to 0.41 mL (Table 4), which represented 3.5 to 18.2% of the bacterial medium added to each vial. Considering the very small volumes of soil extract and fertilizer solution added to each sample incubation vial, there was good agreement among the average Mass 44 peak areas for these samples as well as those for the analyzed international NO_3^- standards (Table 4). The blank 2 M KCl solution (after dispensing, shaking, filtering, etc.) used for extraction had an average Mass 44 peak area of $0.369 \pm 0.025 \text{ Vs}$ ($n = 3$). This represented <1.5% of the average area Mass 44 measured for all the soil extracts (Table 4). Hence, any trace of NO_3^- present within the 2 M KCl solution used for the extraction is negligible, and did not affect the isotopic measurements.

Table 4. Concentrations of NH_4^+ -N and NO_3^- -N, volume added to 20-mL headspace analysis vial, area of Mass 44, and corrected $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of NO_3^- for the unfertilized soil, fertilized soil, applied fertilizer, blank (or 2 M KCl solution), and international NO_3^- standards.

Sample description	NH_4^+ -N	NO_3^- -N	Added volume	Area of Mass 44	Corrected $\delta^{15}\text{N}_{\text{nitrate}}$	Corrected $\delta^{18}\text{O}_{\text{nitrate}}$
	mg L ⁻¹		mL	Vs	‰	
<u>International standards</u>						
IAEA-NO3††	–	0.7	1.01	27.626	4.7	25.6
USGS-34†	–	0.7	1.01	27.488	–2.0	–27.9
USGS-35	–	0.8	0.85	27.095	3.6	56.8
USGS-35	–	0.8	0.85	26.719	3.8	57.7
<u>Blank (idem 2 M KCl)</u>						
Blank 1	0.0	0.0	0.15	0.341	–	–
Blank 2	0.0	0.0	0.15	0.383	–	–
Blank 3	0.1	0.0	0.15	0.384	–	–
Mean	0.0	0.0	–	0.369	–	–
SD	0.0	0.0	–	0.025	–	–
<u>Unfertilized soil</u>						
Plot 1	0.7	1.7	0.41	28.465	5.9	–5.7
Plot 2	0.7	2.5	0.28	26.473	7.0	–6.4
Plot 3	0.7	2.7	0.26	27.842	6.4	–6.6
Mean	0.7	2.3	–	27.593	6.4§	–6.2§
SD	0.0	0.5	–	1.019	0.6	0.4
<u>Fertilized soil</u>						
Plot 1	2.1	6.5	0.11	30.488	0.4	9.1
Plot 2	3.9	9.2	0.08	33.449	–1.6	13.4
Plot 3	2.4	9.0	0.08	31.146	0.9	10.4
Mean	2.8	8.2	–	31.694	–0.1§	11.0§
SD	1.0	1.5	–	1.555	1.3	2.2
<u>Applied fertilizer</u>						
Sample 1	8.3	6.8	0.10	28.314	3.3	25.4
Sample 2	8.5	7.0	0.10	28.329	3.2	25.8
Sample 3	8.3	7.0	0.10	26.963	3.3	25.2
Mean	8.4	6.9	–	27.869	3.3§	25.4§
SD	0.1	0.1	–	0.784	0.1	0.3

† Used to correct the $\delta^{15}\text{N}$ values.

‡ Used to correct the $\delta^{18}\text{O}$ values.

§ Based on a means comparison using an all-pairs Tukey–Kramer test, fertilized soil, unfertilized soil, and fertilizer were characterized by significantly different (at the 0.05 level) $\delta^{15}\text{N}_{\text{nitrate}}$ and $\delta^{18}\text{O}_{\text{nitrate}}$ values.

Fig. 1. Values of $\delta^{18}\text{O}_{\text{nitrate}}$ vs. $\delta^{15}\text{N}_{\text{nitrate}}$ for fertilizer (open diamonds), fertilized soil (black circles), and unfertilized soil (black triangles) from the irrigated cropping systems study designated Rotation U located at Agriculture & Agri-Food Canada's Research Centre in Lethbridge, AB, Canada, determined using the denitrifier technique. Also shown are typical isotopic ranges of NO_3^- derived from NH_4NO_3 fertilizers and soil organic N (after Mengis et al. [2001] and references therein). Note that the range given for soil organic-N-derived NO_3^- is based on the analysis of soil water samples. Also note that Mayer et al. (2004) determined $\delta^{15}\text{N}$ values of synthetic NO_3^- fertilizer as high as 3.2‰, and Kendall (1998) lists $\delta^{18}\text{O}$ values of NO_3^- from soil nitrification as low as -10‰.

The average $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values were $3.3 \pm 0.1\text{‰}$ ($n = 3$) and $25.4 \pm 0.3\text{‰}$ ($n = 3$), respectively, for the applied fertilizer; $-0.1 \pm 1.3\text{‰}$ ($n = 3$) and $11.0 \pm 2.2\text{‰}$ ($n = 3$), respectively, for the fertilized soil; and $6.4 \pm 0.6\text{‰}$ ($n = 3$) and $-6.2 \pm 0.4\text{‰}$ ($n = 3$), respectively, for the unfertilized soil (Table 4). The USGS-35 standard had average $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of 3.7 and 57.3‰ ($n = 2$, Table 4), respectively, which agree with the values shown in Table 3. The $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of NO_3^- determined for the applied fertilizer and the unfertilized and fertilized soil extracts are consistent with published $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values for synthetic fertilizers and NO_3^- derived from soil organic N (Fig. 1, after Mengis et al. [2001] and references therein). The range given in Fig. 1 for NO_3^- derived from soil organic N is based on the analysis of soil water samples. Note that Mayer et al. (2004) determined $\delta^{15}\text{N}$ values of synthetic NO_3^- fertilizer used in Alberta that were as high as 3.2‰, and Kendall (1998) mentioned that the $\delta^{18}\text{O}$ values of NO_3^- derived from soil nitrification may be as low as -10‰. On a $\delta^{18}\text{O}$ vs. $\delta^{15}\text{N}$ plot, the data for each group of samples (fertilizer, fertilized soil, and unfertilized soil) form distinct clusters (Fig. 1). A mean comparison using the all-pairs Tukey-Kramer test between the three groups indicates that all three groups are characterized by significantly different $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of NO_3^- at the 0.05 significance level (Table 4). The greater variability observed in the $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values for the fertilized soil compared with the unfertilized soil was also observed in the NO_3^- -N and NH_4^+ -N concentrations (Table 4). This may be attributed to spatial variability in the distribution of the fertilizer, which had been applied only 2 d before soil sampling.

Interpretations of the $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of the KCl-extractable soil NO_3^- may be compromised by the presence of soil microbes or other soil constituents that were potentially extracted

together with the NO_3^- . For instance, denitrifiers are abundant within the soil microbial community, and thus there might be enough N_2O reductase to reduce some of the NO_3^- -derived N_2O to N_2 . This could increase $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values in the residual N_2O and thus lead to errors in estimating the isotopic signatures of the sample NO_3^- . On closer observation, however, potentially extracted soil microorganisms did not appear to appreciably affect the determination of the $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of KCl-extractable soil NO_3^- using the denitrifier method. First, the volume of soil extract solution added to an analysis vial was much lower than the bacterial medium solution within the vial. Second, the Mass 44 peak areas for the soil extracts were nearly identical to those recorded for the dissolved standards. This suggests that the entire amount of NO_3^- from each soil extract (50 nmol) was converted to N_2O , and was not further reduced to N_2 . If the latter had occurred, then Mass 44 peak areas for the soil extracts should have been smaller than those for the international NO_3^- standards. Third, the $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values obtained are consistent with published data (Fig. 1). Fourth, excellent reproducibility was observed among the δ values for replicate plots, considering inherent variability among soil properties and microbial processes in the field (e.g., Parkin, 1993). Fifth, a clear difference in the N and O isotopic composition of NO_3^- in unfertilized and fertilized soils was observed. The only major difference between the treatments was the addition or omission of synthetic fertilizer.

Another potential negative effect on the determination of the isotopic composition of NO_3^- using the denitrifier method might be the presence of NH_4^+ within a sample and its interaction with the bacterial medium. A test was made in which the international standard IAEA-N1, an ammonium sulfate compound, was analyzed as an individual sample and in a mixture with IAEA-NO3 (data not shown). The IAEA-N1 did not yield a signal, and the $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of the mixture were what would be expected for IAEA-NO3 alone. Thus, the presence of NH_4^+ in a sample does not compromise the performance of the denitrifier method for NO_3^- .

Ideally the $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values obtained for the soil extracts and fertilizer would have been compared with data obtained using an independent method. Unfortunately, we are unaware of an alternate method for reliably estimating the $\delta^{18}\text{O}$ values of KCl-extractable soil NO_3^- . The denitrifier method has, however, been compared with the AgNO_3 method using a set of shallow groundwater samples, whose water type ranged from fresh to brackish, from the same study site (unpublished data, 2006). The data from that study indicated that both methods yielded similar results for the $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of groundwater NO_3^- .

Our results provide an example of how synthetic fertilizer might influence the N and O isotopic composition of KCl-extractable soil NO_3^- . Addition or omission of the NH_4NO_3 fertilizer was the main treatment difference. The shift toward lower $\delta^{15}\text{N}$ and higher $\delta^{18}\text{O}$ values in the fertilized compared to the unfertilized soil can be attributed to fertilization. The data from the fertilized soil fell within the range of values expected for NO_3^- derived from NH_4^+ and NO_3^- in NH_4NO_3 . In addition to nitrification of fertilizer NH_4^+ , various soil microbial processes may also have contributed to the observed changes in the $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values, but the overriding contributor simply was addition of the fertilizer itself. A detailed analysis of processes, such as mineralization-immobilization turnover (Jansson and Persson, 1982), may become important in the longer term but was beyond the scope of this study.

Finally, this short example confirms that the denitrifier technique can be used to simultaneously determine both the $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of soil NO_3^- extracted by 2 M KCl, and can potentially provide essential information on soil N dynamics.

CONCLUSIONS

Our aim was to test whether the denitrifier method can be applied to 2 M KCl soil extracts to determine the $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of NO_3^- . For NO_3^- standards, the standard deviations of the means for replicate analyses did not exceed 0.2‰ for $\delta^{15}\text{N}$ or 0.3‰ for $\delta^{18}\text{O}$ values. After appropriate corrections, differences between the measured and given $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of the NO_3^- standards generally were within the standard deviations for the assigned values. Both $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values were reproducible among contrasting analytical runs. Furthermore, the denitrifier method was successfully applied to NO_3^- in genuine 2 M KCl extracts from fertilized and unfertilized soils. Both soils were characterized by distinct $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values that reflected the addition of the inorganic fertilizer. Hence, the data from this study indicate that the denitrifier method provides a fast, reliable, precise, and accurate way of simultaneously analyzing the natural abundances of ^{15}N and ^{18}O in 2 M KCl-extractable soil NO_3^- .

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