Oxygen exchange with water alters the oxygen isotopic signature of nitrate in soil ecosystems

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ABSTRACT

Combined oxygen (O) and nitrogen (N) stable isotope analyses are commonly used in the source determination of nitrate (NO$_3$). The source and fate of NO$_3$ are studied based on distinct O and N isotopic signatures ($^{18}$O and $^{15}$N) of various sources and isotopic effects during NO$_3$ transformation processes, which differ between sources like fertilizer, atmospheric deposition, and microbial production (nitrification). Isotopic fractionation during production and consumption of NO$_3$ further affects the $^{18}$O and $^{15}$N signal. Regarding the $^{18}$O in particular, biochemical O exchange between O from NO$_3$ and H$_2$O is implicitly assumed not to affect the $^{18}$O signature of NO$_3$. This study aimed to test this assumption in soil-based systems. In a short (24 h) incubation experiment, soils were treated with artificially $^{18}$O and $^{15}$N enriched NO$_3$. Production of NO$_2$ from nitrification during the incubation would affect both the $^{18}$O and the $^{15}$N enrichment. Oxygen exchange could therefore be studied by examining the change in $^{18}$O relative to the $^{15}$N. In two out of the three soils, we found that the imposed $^{18}$O enrichment of the NO$_3$ declined relatively more than the imposed $^{15}$N–NO$_2$ enrichment. This implies that O exchange indeed affected the O isotopic signature of NO$_3$, which has important implications for NO$_3$ source determination studies. We suggest that O exchange between NO$_3$ and H$_2$O should be taken into consideration when interpreting the O isotopic signature to study the origin and fate of NO$_3$ in ecosystems.

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1. Introduction

Increasing concentrations of nitrate (NO$_3$) in ground- and surface water constitute an important environmental concern. They are generally linked to increased use of nitrogen (N) fertilizer and animal manure, atmospheric deposition and discharge of sewage waste (Galloway et al., 2003; Howarth et al., 1996). Anthropogenic input of nitrogen into our environment is largely used to evaluate the source and fate of NO$_3$ through biological processes. However, the relative importance of different NO$_3$ sources remains difficult to quantify.

Analysis of the $^{15}$N and $^{18}$O signatures of NO$_3$ is increasingly used to evaluate the source and fate of NO$_3$ in ecosystems including groundwater, drainage water, and river catchments (Amberger and Schmidt, 1987; Burns et al., 2009; Burns and Kendall, 2002; Durka et al., 1994; Kendall et al., 2007; Wankel et al., 2006). Different sources and processes are assumed to lead to distinct isotopic signatures in NO$_3$ (Fig. 1); atmospheric deposition and synthetic fertilizers are relatively highly enriched in $^{18}$O; NO$_3$ produced from organic fertilizer (manure, slurry) has relatively high $^{15}$N values; denitrification leaves the residual NO$_3$ pool relatively enriched in both isotopes, and; NO$_3$ produced from nitrification of soil N typically results in the lowest $^{15}$N and $^{18}$O signatures. The expected range of the $^{18}$O of NO$_3$ from nitrification is derived from the relative contribution of O$_2$ and H$_2$O to the total O incorporated during the oxidation steps (Fig. 1). The O$_2$ contributes one atom during ammonia oxidation to hydroxylamine (NH$_2$OH), and H$_2$O contributes the other two O atoms during further oxidation to nitrite (NO$_2$) and NO$_3$ (Andersson and Hooper, 1983; Hollocher, 1984; Hollocher et al., 1981; Kendall et al., 2007). Atmospheric O$_2$ has a relatively large $^{18}$O ($+23.5\,^{\circ}\text{SMOW}$), and the $^{18}$O of O$_2$ in soil may be even more enriched due to fractionation by respiration in soil (Guy et al., 1993; Kendall et al., 2007). The relatively low $^{18}$O of soil H$_2$O ($-25$ to $+4\,^{\circ}\text{SMOW}$, Amberger and Schmidt, 1987) explains the relatively low $^{18}$O of NO$_3$ from nitrification compared to the other sources (Fig. 1).
In multiple NO₃ source determination studies the δ¹⁸O value of NO₃ is low compared to the δ¹⁸O value of atmospheric deposition and (nitrate-) fertilizer, and closer to the range expected from biologically produced NO₃ from nitrification of ammonium fertilizers (-5 to -15‰SMOW, Fig. 1). It is consequently reasoned that most NO₃ in these systems (e.g. groundwater, drainage water, or river catchments) is derived from microbial nitrification within the soil system (Barnes and Kendall, 2002; Mayer et al., 2002; Spoelstra et al., 2001; Williard et al., 2001). Casciotti et al. (2002) already suggested that nitrifiers may not always use O from O₂ and H₂O in this 1:2 ratio, with a potentially greater influence of the δ¹⁸O of water on NO₃. However, oxygen exchange between H₂O and NO₃ is in general hardly considered as a factor contributing to the δ¹⁸O of the NO₃ pool. As mentioned earlier, soil water has the lowest δ¹⁸O compared to all the above mentioned NO₃ sources, including nitrification-derived NO₃ which is assumed to still obtain part of its O from O₂ (at approximately +23.5‰SMOW, Fig. 1). If exchange of O between H₂O and NO₃ would indeed occur, it would lower the δ¹⁸O signature of the NO₃ pool. Regardless of the original source of the NO₃, its final ¹⁸O isotopic signature would partly be defined by the δ¹⁸O signature of H₂O, depending on the extent of the O exchange between NO₃ and H₂O. As a consequence, the contribution of nitrification NO₃ would then be overestimated at the expense of atmospheric deposition and fertilizer input.

Isotope fractionation during denitrification leaves the residual NO₃ relatively enriched in ¹⁵N and ¹⁸O, which is used to study the progress of denitrification (e.g. Aravena and Robertson, 1998; Büttcher et al., 1990; Groffman et al., 2006; Panno et al., 2006; Wassenaar, 1995). Again, the occurrence of O exchange could lower the δ¹⁸O–NO₃ value, since H₂O is depleted in ¹⁸O compared to NO₃. Such a lowered δ¹⁸O–NO₃ value would partially mask the enrichment effect of denitrification. As a result, the rate of denitrification would be underestimated.

In summary, multiple sources and processes have to be taken into account to evaluate the fate and origin of NO₃, based on the O isotopic signature. However, of all the defining factors of the δ¹⁸O–NO₃ the potential effect of O exchange with H₂O has not been studied. Studies have previously identified that O exchange during denitrification of NO₃ to nitrous oxide (N₂O) affects the O isotopic signature of the produced N₂O (Kool et al., 2009b). Additionally, nitrifiers may also mediate O exchange during N transformation processes (Casciotti et al., 2010; Kool et al., 2009a). If the occurrence of O exchange also alters the δ¹⁸O of the NO₃ in ecosystems significantly, it would reduce the accuracy of the δ¹⁸O isotopic signature as indicator of the sources of NO₃ (e.g. Barnes and Raymond, 2010; Kool et al., 2007).

The aim of this research was to investigate the potential occurrence and effect of O exchange on the O isotopic signature of the NO₃ pool in actual ecosystems. With the uncertainty about the exact isotopic signature of the sources and the isotopic fractionation factors of NO₃ production and consumption at natural abundance, a potential effect of O exchange cannot easily be distinguished at natural abundance. Therefore, to investigate the potential occurrence and effect of O exchange on the O isotopic signature of NO₃, we carried out a soil incubation experiment in which we used NO₃ that was artificially enriched with ¹⁸O and ¹⁵N. By tracing the fate of ¹⁸O relative to ¹⁵N enrichment of NO₃ we studied O exchange as a potential defining factor of the O isotopic signature of NO₃.

2. Materials and methods

2.1. Experimental set-up

To include diversity in soil types, three soils under different management regimes were used in our experiment: two silt loam grassland soils from experimental field Easter Bush near Edinburgh (UK) that differed in fertilizer and grazing intensity (i.e. ‘moderate’ and ‘intensive’ management; Gm and Gi respectively), and one relatively poor, arable sandy soil from experimental farm ‘Droevedaal’ near Wageningen, The Netherlands (A). Soils were dried at 40 °C, sieved (2 mm) for homogeneity and stored at 4 °C until further use. Soil samples of 100 g soil per jar were pre-incubated for seven days at 40% water holding capacity (WHC) and 16 °C in glass jars. At the start of the incubation samples were treated with 50 mg NO₃-N kg⁻¹ soil. The applied NO₃ was artificially enriched in ¹⁸O at 2.0 atom% excess (TR1) or ¹⁵N at 30.0 atom% excess (TR2). The treatment solutions were prepared from ¹⁸O-labeled NO₃ of 75 atom% excess (CKGas Ltd., Hook-Hampshire, UK) and ¹⁵N-labeled NO₃ of 98 atom% excess (Chemotrade mbH, Düsseldorf, GE). In combination with non-enriched compound the desired enrichment of the treatment solution was established. The NO₃ was applied as Ca(NO₃)₂·4H₂O (¹⁵N enriched and non-enriched) and partially as NaN₃ in TR2 (¹⁸O-enriched NO₃). Moisture content was raised to 80% WHC with the application of the NO₃ at the start of the incubation period. Treatments were replicated four times for two sampling moments, resulting in a total of 48 units (three soils, two labeling treatments, two sampling moments, four replicates).

Following the application of labeled NO₃, soil was (destructively) sampled for the time zero (t0) measurement as soon as possible (within 4 h after application at the latest). At the end of the 24 h incubation period, the t24 samples were collected. Soil was thoroughly mixed (in the same manner for all samples) to ensure a representative homogenized subsample was taken from each jar. The NO₃ in the soil samples was extracted using a KCl solution (20 g moist soil with 50 ml 1 M KCl) immediately after sampling. The O isotopic enrichment (δ¹⁸O) of the NO₃ in TR1 was determined in the KCl extracts by the denitrifier method (Casciotti et al., 2002; Xue et al., 2010), in which the sample NO₃ is converted to N₂O gas which is then analyzed for its isotopic signature. The N₂O is corrected similarly as outlined for nitrogen by the equations of Stevens et al. (1993). Measured values are standardized using a multi-point calibration of nitrate standards supplied by NIST (National Institute
of Standards and Technology, Gaithersburg, MD, U.S.A.). For the TR2 samples, the $^{15}$N isotopic signature of the NH$_4^+$ and NO$_3^-$ were measured using the microdiffusion technique, modified from Stark and Hart (1996) (Kool et al., 2009a). Analyses of the $^{15}$N isotopic signature of NH$_4^+$ were included to check for the absence of a significant effect of DNRA or immobilization followed by remineralization of $^{15}$N–NO$_3^-$ resulting in newly produced, $^{15}$N enriched NH$_4^+$. Isotopic analyses were carried out at the UC Davis Stable Isotope Facility, at Davis (CA), USA.

2.2. Data evaluation

The advantage of the use of enriched compounds is that the imposed enrichment will exceed any naturally occurring fractionation that may be going on. The enrichment is expressed as atom% excess, i.e. the percentage of the element present as the specified isotope (atom%) in excess relative to the atom% of the background. For example for $^{15}$N:

$$\text{atom}\% \text{ excess} = \frac{\text{atom}\% ^{15}\text{N}_{\text{sample}} - \text{atom}\% ^{15}\text{N}_{\text{background}}}{\text{atom}\% ^{15}\text{N}_{\text{background}}} \times 1000.$$ 

Natural abundance variations are much smaller, and are therefore regularly expressed in delta units ($\delta$, $\delta_{\text{me}}$), which denote the relative deviation from the background atom% of a reference standard:

$$\delta ^{15}\text{N}_{(\text{me})} = 1000 \times \left(\frac{\text{atom}\% ^{15}\text{N}_{\text{sample}}}{\text{atom}\% ^{15}\text{N}_{\text{standard}}} - 1\right).$$

When expressed as atom%, natural abundance isotope effects would be in the order of promilles. Therefore, with the use of enriched compounds, fractionation effects during production and consumption of NO$_3^-$ will become negligible.

The $^{18}$O and $^{15}$N enrichment of NO$_3^-$ at t24 were compared to the $^{18}$O and $^{15}$N enrichment at t0. Changes in the $^{18}$O and $^{15}$N enrichment over the incubation period were identified using t-tests; significant differences were identified at $P < 0.05 \ (\alpha = 0.05)$.

However, a decline in the O isotopic enrichment over time alone does not conclusively identify O exchange. Newly produced NO$_3^-$ would obviously also dilute the enrichment. However, a dilution of the (intermediate) compounds would affect both O and N enrichments equally, and therefore would not change their ratio. This ratio will however decrease when O exchange occurs, as that would only alter the $^{18}$O enrichment and not the $^{15}$N. In other words, regardless of production of new NO$_3^-$, the ratio of the $^{18}$O to the $^{15}$N enrichments should be conserved over time in the absence of O exchange. Therefore, to prove the presence of O exchange, we determined whether the $^{18}$O and $^{15}$N enrichment had changed relatively to each other during the incubation, i.e. we evaluated the $^{18}$O/$^{15}$N ‘Enrichment Ratio’ of the NO$_3^-$ ($\text{ERR}_{\text{NO$_3^-$}}$). The ratio at t24 ($\text{ERR}_{\text{NO$_3^-$}}(t24)$) was compared to the ratio at t0 ($\text{ERR}_{\text{NO$_3^-$}}(t0)$), defining the enrichment ratio retention $\text{ERR}_{\text{NO$_3^-$}}$:

$$\text{ERR}_{\text{NO$_3^-$}}(\%) = 100 \times \frac{\text{ERR}_{\text{NO$_3^-$}}(t24)}{\text{ERR}_{\text{NO$_3^-$}}(t0)}$$

with $\text{ERR}_{\text{NO$_3^-$}} = \frac{^{18}\text{O}(\text{NO$_3^-$})/^{15}\text{N}(\text{NO$_3^-$})}{^{15}\text{N}(\text{NO$_3^-$})/^{15}\text{N}(\text{NO$_3^-$})}$.

Where $^{18}\text{O}(\text{NO$_3^-$})$ and $^{15}\text{N}(\text{NO$_3^-$})$ are the $^{18}$O and $^{15}$N isotopic enrichments of the NO$_3^-$ in TR1 and TR2 respectively, determined at t0 for $\text{ERR}_{\text{NO$_3^-$}}(t0)$ or at t24 for $\text{ERR}_{\text{NO$_3^-$}}(t24)$. As the $\text{ERR}_{\text{NO$_3^-$}}$ are ratios, it is not possible to obtain standard errors of the means. Standard errors of the $\text{ERR}_{\text{NO$_3^-$}}$ were therefore approximated by a first-order Taylor linearization (Kendall and Stuart, 1977; Kool et al., 2010).

With the use of enriched compounds, the fractionation effects during production and consumption of NO$_3^-$ became negligible. Therefore, in the absence of O exchange the $\text{ERR}_{\text{NO$_3^-$}}$ should not change over the course of the incubation, i.e. the $\text{ERR}_{\text{NO$_3^-$}}(t24)$ should be 100%. O exchange would cause a decrease in the $\text{ERR}_{\text{NO$_3^-$}}$ at t24 compared to t0, represented by a loss in the $\text{ERR}_{\text{NO$_3^-$}}$.

3. Results and discussion

Our results show that in all soils the $^{18}$O enrichment of NO$_3^-$ decreased significantly over the course of the incubation time (24 h), while the $^{15}$N enrichment of NO$_3^-$ did not (Fig. 2, Table 1). Moreover, the $\text{ERR}_{\text{NO$_3^-$}}(t24)$ was approximately 80, 88, and 96% for Gm, Gi and A, respectively, confirming a loss in $^{18}$O relative to the $^{15}$N (Fig. 3). Results of all data analyses on the isotopic enrichments and the $\text{ERR}_{\text{NO$_3^-$}}$ are presented in Table 1. Overall small standard errors support high accuracy of the isotopic enrichment data. One outlying data point for soil A at t0 (one replicate of the $^{15}$N–NO$_3^-$ treatment) was excluded from the data calculations in Figs. 2 and 3.

Data analysis showed that also when including this data point, the $^{15}$N of the NO$_3^-$ did not change significantly over the incubation period whereas the $^{18}$O–NO$_3^-$ did (Table 1). However, as the $\text{ERR}_{\text{NO$_3^-$}}$ exceeds 100% when this data point is included, the presence of O exchange was not convincingly confirmed in this soil (A) (Table 1).

Our results suggest that O exchange affected the O isotopic signature of NO$_3^-$ in at least two of the three soils tested. To our knowledge, no soil-based study has experimentally identified the effect of O exchange on the O isotopic signature of NO$_3^-$ Given our short incubation period, O exchange may likely be even more pronounced in actual ecosystems. Loss in the $^{18}$O relative to the $^{15}$N enrichment of NO$_3^-$ could also result from dissipatory nitrate reduction to ammonium (DNRA) or immobilization and remineralization followed by nitrification, as in the ‘immobilization–mineralization turnover concept’ suggested by Mengis et al. (2001). Our incubation period was kept deliberately short to exclude significant effects of these processes. Our $^{15}$N–NH$_4^+$ analyses indeed confirmed that DNRA and/or immobilization plus mineralization did not enrich our NH$_4^+$ pool to such extent that NO$_3^-$ recycling through these routes followed by nitrification would have contributed significantly to the observed loss in the (relative) $^{18}$O enrichment of NO$_3^-$ (data not shown).

![Fig. 2. The O and N isotopic signatures of NO$_3^-$ (in TR1 and TR2, respectively) for t0 and t24. In all three soils (Gm, Gi and A), the differences between isotopic signatures at t0 and t24 were significant for $^{18}$O and insignificant for $^{15}$N. T-test values are reported next to the data. Dashed lines with open symbols represent $^{18}$O data, solid lines with closed symbols represent $^{15}$N. The error bars denote the standard errors ($n = 4$ for all, except for $^{15}$N at t0 of soil A; $n = 3$).](image-url)
remained under approximately 0.5 at% excess at both t0 and t24 in all samples).

As we study the process of O exchange in our soil incubations, we should also consider whether O exchange may have occurred after the incubation in our samples, e.g., during KCl extraction, storage and transport, as well. During sample storage or transport, O exchange was likely minimal due to the high salt concentrations in the sample (KCl extracts) that inhibited microbial activity. Also, during the analytical procedure of the denitrifier method O exchange is assumed to be minimal: it is one of the selection criteria for the denitrifier strain used in this technique (Pseudomonas aerofaciens) (Casciotti et al., 2002). Moreover, Rock and Ellert (2007) showed that this method could also be used to analyze KCl-extractable NO3, and it has shown excellent repeatability and good comparison with the silver nitrate method (Xue et al., 2010). Most importantly however, we can disregard this uncertainty in our approach as such undesired O exchange would not affect our results. Because the 18O and 15N enrichments were measured at both the beginning and end of the incubation, any O exchange after sampling that would affect the obtained 18O signature would therefore have likely affected all measurements. O exchange after sampling would thereby not interfere with our evaluation of the occurrence of O exchange during the incubation between measurements periods.

If O exchange is indeed a defining factor of the O isotopic signature of NO3, this phenomenon would have significant implications for source determination of NO3. The quantitative contribution of the processes defining the O isotopic signature all show uncertainties: the isotopic signatures of the different sources and the extent of isotopic fractionation effects during production and consumption of NO3 are not exactly known, but only within a certain range (Bedard-Haughn et al., 2003; Kendall et al., 2007). Results from experimental studies at natural abundance can therefore generally be explained within the available conceptual framework, and will not directly lead to suspicion of O exchange without the use of artificially enriched compounds.

Observed discrepancies in the δ18O signature of NO3 (both higher and lower) compared to the expected δ18O based on reaction stoichiometry have previously been attributed to fractionation, microscale variability in 18O of O2 and H2O, and contributions of heterotrophic nitrifiers whose nitrifying mechanism may differ with respect to O incorporation (Burns and Kendall, 2002; Kendall et al., 2007; Mayer et al., 2001). Mengis et al. (2001) derived from the 15N signatures of the soil NO3 that fertilizer was the major source of the NO3. However, the observed 18O–NO3 signatures were significantly lower than that of the fertilizer. They suggest that the original 18O of the fertilizer NO3 in their agricultural soils was masked by a recycling of the NO3 through microbial immobilization followed by mineralization and nitrification. Our results show that O exchange may provide an alternative explanation for such a discrepancy between the 18O and 15N signatures of NO3 of the soil compared to its potential sources.

The presence of O exchange should not be completely unexpected when considering the relevant biogeochemical pathways. First, O exchange may occur during nitrification, i.e. the production of NO3. The final nitrification step of NO3 production from NO2 oxidation incorporates H2O–O which is catalyzed by nitrite oxidoreductase (Aleen et al., 1965; Rokk et al., 1986). However, the reduction of NO3 to NO2 is found to be controlled by the same enzyme (Sundermeyer-Klinger et al., 1984; Wood, 1986). Hence, this process is reversible, which may provide the mechanistic explanation of the O exchange. If forward and reverse NO3/NO2 transformations take place concurrently, the involvement of H2O–O in this transformation implies that all O in NO3 (and NO2) can ultimately be replaced by O from H2O.

Furthermore, in the first steps of nitrification (ammonia oxidation to NO2) microbially mediated exchange may occur between H2O and nitrite (NO2). Early studies already reported O exchange in pure cultures of nitrifiers, both ammonia and nitrite oxidizers (Andersson et al., 1982; DiSpirito and Hooper, 1986; Kumar et al., 1983). In recent studies on pure cultures of four different strains of ammonia oxidizing bacteria (AOB) Casciotti et al. reported O exchange of 1–25% of the NO2–O atoms (Casciotti et al., 2010). As a result of O exchange, microbially produced NO3 and NO2 will exhibit δ18O values that are more close to the δ18O of H2O than expected based on reaction stoichiometry. Using a multi-box model to evaluate the δ18O–NO3 in the ocean, Sigman et al. (2009) also suggested that as a result of O exchange less than one out of six of the O atoms in NO3 originates from O2, which is consistent with at least a 50% exchange of O. Casciotti et al. (2010) noted the
discrepancy between their observed relatively low amounts of exchange (maximally 25%) compared to these model derived exchange rates. They suggest this could in part be explained by a possibly significant contribution of ammonia oxidizing Archaea (AOA) to total nitrification in ocean waters, because AOA may perform nitrification with alternative reaction mechanisms and enzymes. However, it has not been considered whether, besides during NO3 production, the O isotopic signature of NO3 is affected by O exchange during the consumption of NO2.

Oxygen exchange during reduction of NO3 has clearly been shown to affect the O isotopic signature of the product nitrous oxide (N2O) (Kool et al., 2009b). Pure culture studies on denitrifying bacteria have also shown O exchange to affect the substrate NO2 (Garber and Hollocher, 1982; Shearer and Kohl, 1988). In the stepwise reduction of NO3 to N2O the O exchange is suggested to be mainly associated with the NO3 and NO reduction steps (Garber and Hollocher, 1982; Kool et al., 2007). Again, the reversibility of the reduction steps may explain the potential occurrence of O exchange with H2O. In environmental studies an O isotope effect of O exchange on the substrate NO3 during its reduction is generally not considered or assumed to be extremely slow (Bohlke et al., 2003). Only in a recent pure culture study on two denitrifying strains, Knöller et al. (2010) showed that while the N isotope signature of the NO3 bulk largely followed the dynamics of fractionation, the O isotope values were evidently influenced by isotope equilibration with O of the ambient H2O. Notably, for sulfate (SO42−) it is more generally acknowledged that the 18O is affected not only by isotope fractionation, but also by equilibration with H2O (Böttcher and Thamdrup, 2001; Farquhar et al., 2008; Fritz et al., 1989; Turchyn et al., 2010). Oxygen and sulfur isotope effects for SO42− during its bacterial reduction process were modeled by Brunner and Bernasconi (2005) and Brunner et al. (2005), incorporating the forward and reverse steps in the reduction. Using this model, observed patterns in isotope data from natural environments and laboratory studies could be better explained. Analogously for reduction of NO3, such a model based on O isotope exchange effects may better predict O isotope effects on NO3 in natural conditions as well as pure cultures (Brunner et al., 2005; Knöller et al., 2010).

4. Conclusion

Our experiment indicates that O exchange may indeed affect the O isotopic signature of NO3 in soil ecosystems. Studies evaluating the source and fate of NO3 in ecosystems based on its O isotopic signature may have been overestimating microbial nitrification as a source of NO3, at the expense of (anthropogenic) sources like fertilizer and atmospheric deposition. It may also have led to underestimation of the progress of denitrification. Our findings should instigate further and more elaborate ecosystem-based studies to identify the presence and effect of O exchange on the O isotopic signature of NO3. Ultimately, taking O exchange into account as a defining factor of the O isotopic signature of NO3 will improve the interpretation of O isotopic analyses resulting in more accurate determination of the source of NO3 in ecosystems.

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