Oxygen Isotopic Composition of Nitrate Produced by Freshwater Nitrification

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Oxygen Isotopic Composition of Nitrate Produced by Freshwater Nitrification

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Oxygen Isotopic Composition of Nitrate Produced by Freshwater Nitrification

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Abstract

Identifying sources of nitrate ($\text{NO}_3^-$) in the environment is important to elucidate causes of water quality impairment and eutrophication. Measurements of naturally occurring stable isotope ratios of nitrogen ($^{15}\text{N}/^{14}\text{N}$) and oxygen ($^{18}\text{O}/^{16}\text{O}$) in NO$_3^-$, can be used to determine the sources, dispersal, and fate of natural and contaminant NO$_3^-$ in aquatic environments. To this end, it is necessary to know how NO$_3^-$ isotopologues are modified by biological reactions, as heavy and light isotopes have different reaction rates. One important microbial reaction that influences isotope ratios of NO$_3^-$ in the environment is nitrification, the biological oxidation of ammonium (NH$_4^+$) to nitrite (NO$_2^-$) then NO$_3^-$, the influence of which is not well understood in freshwater systems. The purpose of this study was to determine the influence of the $\delta^{18}\text{O}$ of ambient water on the isotopic composition of NO$_3^-$ produced by freshwater nitrification. Water was collected from two streams in New England during the fall and spring, which were amended with NH$_4^+$ and with increments of $^{18}\text{O}$-enriched water, and then monitored the isotopic composition of NO$_2^-$ and NO$_3^-$ produced by natural consortia of nitrifiers. Although oxidation rates differed between the two stream waters, the final $\delta^{18}\text{O}$ of NO$_3^-$ produced in both experiments revealed a sensitivity to the $\delta^{18}\text{O}$ of water mediated by (a) isotopic equilibration between water and NO$_2^-$ and (b) kinetic isotope fractionation during O-atom incorporation from molecular oxygen and water into NO$_2^-$ and NO$_3^-$: Our results concur with seawater incubations and nitrifying culture experiments that have demonstrated analogous sensitivity of the $\delta^{18}\text{O}$ of nitrified NO$_3^-$ to equilibrium and kinetic O isotope effects (Buchwald et al. 2012). These findings have important implications for interpretations of O isotopes in NO$_3^-$ source apportionation studies.
1. Introduction

Human activity has greatly altered the nitrogen cycle, particularly in terrestrial, aquatic and coastal ecosystems, through fossil fuel combustion, use of industrial nitrogen fertilizer, and the release of nitrogen in wastewater. Reactive nitrogen fixed industrially by the Haber Bosch process now rivals that introduced to the biosphere by biological nitrogen fixation (Galloway et al., 2004). The release of reactive nitrogen has resulted in deterioration of groundwater quality, and in eutrophication of freshwater, estuaries, and shelf seas around the globe.

Identifying sources of nitrate (NO₃⁻) in the environment is important to elucidate causes of water quality impairment and eutrophication. Measurements of naturally occurring stable isotope ratios of nitrogen (¹⁵N/¹⁴N) and oxygen (¹⁸O/¹⁶O) in NO₃⁻, can be used to determine the sources, dispersal, and fate of natural and contaminant NO₃⁻ in aquatic environments.

Henceforth we express isotope ratios in “delta” notation, where;

\[
\delta^{^{15}N} = \left[\frac{(^{15}N)_{\text{sample}}}{(^{15}N)_{\text{air}}} - 1\right] \times 1000
\]

\[
\delta^{^{18}O} = \left[\frac{(^{18}O)_{\text{sample}}}{(^{18}O)_{\text{VSMOW}}} - 1\right] \times 1000
\]

To use NO₃⁻ isotopes in this way, it is necessary to know the extent to which NO₃⁻ isotopologues, molecules that differ only in their isotopic composition, are influenced by biological reactions that produce and consume NO₃⁻, as heavy and light isotopologues can have different reaction rates during biological transformations. Small differences in respective reaction rates (k) can result in notable isotope fractionation between reactant and product.
The extent to which heavy and light isotopologues are fractionated during a unidirectional chemical reaction is described by kinetic isotope effects, ε, where

$$\varepsilon_{\text{heavy}} = \left( \frac{\text{light}}{\text{heavy}} - 1 \right) \times 1000$$

(Mariotti et al., 1981).

One important microbial reaction that influences isotope ratios of NO$_3^-$ in the environment is nitrification, the biological production of NO$_3^-$ from the oxidation of ammonium (NH$_4^+$) to nitrite (NO$_2^-$) then NO$_3^-$. This process is carried out bacteria and archaea that use NH$_4^+$ (as ammonia, NH$_3$) and NO$_2^-$ as respective reductants to chemosynthesize organic carbon from carbon dioxide (CO$_2$). In the environment, NO$_3^-$ produced by nitrification can be distinguished from other sources, namely from atmospheric deposition or industrial fertilizers, based on its $\delta^{18}$O$_{NO_3}$ composition (Fig. 1). NO$_3^-$ produced by nitrification typically has a comparatively low $\delta^{18}$O relative to atmospheric and fertilizer NO$_3^-$, which is assumed to derive from the fractional contribution of O atoms originating from O$_2$ (~24.2‰; Kroopnick & Craig, 1972) and water (-10 to 0‰) during the biological oxidation of NH$_4^+$ to NO$_3^-$. Indeed, a culture study of NH$_4^+$ oxidizing bacteria using $^{18}$O$_2$ and H$_2^{16}$O enriched medium revealed that one O atom is first incorporated from O$_2$ to produce hydroxylamine (NH$_2$OH) followed by a second
oxygen atom incorporation from H₂O to produce NO₂⁻ (Andersson & Hooper, 1983). A parallel study using NO₂⁻ oxidizing bacteria with experimental treatments of H₂¹⁸O,¹⁸O₂, or P¹⁸O₄ enriched media demonstrated that the third O atom incorporated into NO₃⁻ is sourced from water (Kumar et al., 1983). The δ¹⁸O_NO₃ estimated from the fractional source contribution of O atoms (Eqs. 1 and 2) has thus provided a benchmark from which to identify the contribution of nitrified NO₃⁻ in the environment (Amberger & Schmidt, 1987; Voerkelius et al., 1990; Durka et al., 2004).

\[
\delta^{18}O_{NO_{2, nitrified}} = \frac{1}{2} (\delta^{18}O_{H_{2}O}) + \frac{1}{2} (\delta^{18}O_{O_2}) \tag{1}
\]

\[
\delta^{18}O_{NO_{3, nitrified}} = \frac{2}{3} (\delta^{18}O_{H_{2}O}) + \frac{1}{3} (\delta^{18}O_{O_2}) \tag{2}
\]

By this convention, the δ¹⁸O_NO₃ produced by nitrification in systems where the δ¹⁸O of water ranges from -10 to 0‰ should range between 1.4 and 8.1‰, assuming a δ¹⁸O for molecular O₂ of ~24.2‰ (Kroopnick & Craig, 1972). However, a number of observations from soil incubation experiments suggest that the δ¹⁸O of nitrified NO₃⁻ may not conformed to the model described by Eq. 2. Amberger and Schmidt, 1987 and Voerkelius et al., 1990 reported negative δ¹⁸O values for nitrified NO₃⁻, whereas others reported values greater than expected from Eq. 2. (Burns & Kendall, 2002; Mayer et al., 2001; Spoelstra et al., 2007). Values of δ¹⁸O of nitrified NO₃⁻ lower than expected from Eq. 2 have been ascribed to a biologically mediated exchange of O atoms between NO₂⁻ and H₂O during NH₄⁺ oxidation (Andersson et al., 1982; Andersson & Hooper, 1983; Fang et al., 2012; Snider et al., 2010). Values greater than expected δ¹⁸O for nitrification were attributed to O isotopic enrichments of H₂O and O₂ due to evaporation and respiration.
Mayer et al. 2001 also suggests that only one-third of the oxygen in nitrified NO$_3^-$ was derived from water in a coniferous soil incubation, which they attribute to purported heterotrophic nitrification – in which two of the three O atoms in NO$_3^-$ are allegedly derived from an organic nitrogen compound and only one from water (Wood 1988; Wood 1990; Hollocher 1984). The heterogeneity among observations and explications of observed trends reflect a lack of fundamental understanding of the $\delta^{18}$O variations produced by nitrification, which leads to uncertainty in interpretations of $\delta^{18}$O signatures of NO$_3^-$ in freshwater environments.

Recent evidence from cultures and field incubations, however, reveal that $\delta^{18}$O$_{\text{NO}_3}$ is sensitive not only to $\delta^{18}$O composition of water incorporated, but also to kinetic and equilibrium isotope effects. (Figure 2). The respective incorporations of O atoms from molecular O$_2$ and water during NH$_4^+$ oxidation by cultures of bacterial and archaeal isolates from marine and terrestrial systems are associated with substantial isotope effects ($^{18}e_{k,H2O,1}$ and $^{18}e_{k,O2}$), on the order of 18 to 38‰ combined (i.e. $^{18}e_{k,H2O,1} + ^{18}e_{k,O2}$; Casciotti et al. 2010; Buchwald et al. 2012). Culture work also revealed a pH- and temperature-dependent tendency for biologically enhanced O atom exchange between NO$_2^-$ and H$_2$O during NH$_4^+$ oxidation (0 –
25% of O atoms exchanged over 2 days), with an associated equilibrium isotope effect of \(\sim 13\%\) (Casciotti et al. 2007; Casciotti et al. 2010; Figure 2).

Culture studies on marine \(\text{NO}_2^-\) oxidizing bacteria have also revealed a role for incorporation and kinetic isotope effects during \(\text{NO}_2^-\) oxidation to \(\text{NO}_3^-\). For one, the two O atoms of \(\text{NO}_2^-\) are subject to an inverse kinetic isotope effect upon conversion to \(\text{NO}_3^-\) (\(^{18}\varepsilon_{\text{K,NO}_2}\)), thus causing a depletion of \(^{18}\text{O}\) in the \(\text{NO}_2^-\) pool during oxidation (Casciotti 2009; Buchwald and Casciotti 2010). Another substantial isotope effect was observed in association with O atom incorporation from water into \(\text{NO}_3^-\) (\(^{18}\varepsilon_{\text{K,H}_2\text{O},2}\)), ranging from 9\% to 25\% (Buchwald and Casciotti 2010; Buchwald et al. 2012; Figure 2).

In order to determine if insights from culture studies are pertinent to environmental isotope dynamics, Buchwald et al. 2012 investigated the \(\delta^{18}\text{O}\) value of \(\text{NO}_3^-\) produced by co-cultures of \(\text{NH}_4^+\) oxidizing archaea or \(\text{NH}_4^+\) oxidizing bacteria and \(\text{NO}_2^-\) oxidizing bacteria, as well as by incubations of natural marine assemblages. Examination of the \(\delta^{18}\text{O}\) of \(\text{NO}_2^-\) and \(\text{NO}_3^-\) produced by co-culture and during seawater incubations revealed O atom incorporation isotope effects and \(\text{NO}_2^-\) isotopic equilibration analogous to those observed in monocultures. The results suggest the \(\delta^{18}\text{O}\) of newly produced \(\text{NO}_3^-\) in the ocean (when \(\delta^{18}\text{O}_{\text{H}_2\text{O}} = 0\%\) and \(\delta^{18}\text{O}_{\text{O}_2} = 23.5\%\)) most likely lies between -1.5\% and 1.3\%, much lower than the 8.1\% suggested by Eq. 2.

Accurate interpretation of isotope distribution in the environment requires a sound mechanistic understanding on factors influencing the isotope composition of \(\text{NO}_3^-\) produced by nitrification. While isotope effects and \(\text{NO}_2^-\) equilibration with water have been adroitly documented in monocultures and in incubations of seawater communities, these factors are
often disregarded in studies of freshwater environments. Neglecting exchange and isotope effects can lead to an overestimation of nitrification as a source of NO₃⁻ in source apportioning studies. The purpose of this study is to determine the influence on the δ¹⁸O of ambient water on the isotope composition of NO₃⁻ produced during nitrification in freshwater systems. In particular, we aim to determine whether the δ¹⁸O_NO₃ produced by a natural consortium of freshwater nitrifiers can be described by fractional source contribution (Eq. 2) or if isotope effects and/or NO₂⁻ equilibration need to be considered. In doing so, we aim to bridge the gap between interpretations of the δ¹⁸O_NO₃ produced by nitrification in marine and culture nitrification vs. freshwater.

2. Methods

In order to gauge the potential influence of isotope effects and NO₂⁻ equilibration on the oxygen isotope composition of NO₃⁻ produced by natural communities of nitrifiers, we incubated stream water in incremental δ¹⁸O_H₂O treatments. The evolution of N species and their O isotope composition was monitored as NH₄⁺ was oxidized sequentially from NH₄⁺ to NO₂⁻ and then NO₃⁻, following a protocol analogous to Buchwald et al. 2012 for incubations of natural seawater consortia. Briefly, 40 L of stream water was collected from 2 freshwater streams in coastal Connecticut, in acid-washed 20 L plastic (polypropylene) carboys. The first sampling took place during the Fall of 2016 and will be referred to as Experiment 1, the second sampling occurred in the Spring of 2017 and will be referred to as Experiment 2. Within a few hours of collection, water was returned to the lab and homogenized into one large 50 L, spigoted carboy. A multi-layered coarse mesh was attached to the spigot to filter out large particles as
the water was dispensed into twelve acid-washed, sterile, 2 L glass media bottles. Each bottle was amended with NH₄Cl to obtain a concentration of 50 µmol L⁻¹. The δ¹⁸O_H₂O of each was adjusted in triplicate bottles by adding increasing amounts of 97-atom-% ¹⁸O-labeled water (Cambridge Isotope Laboratories; OLM-240-97) to obtain respective δ¹⁸O_H₂O treatments described by Table 1. δ¹⁸O_H₂O treatments for Experiment 1 all fall within the range of natural abundance water isotopes. The dynamic range of δ¹⁸O_H₂O treatments was broadened for Experiment 2 in order to obtain more accurate estimates of potential isotope dynamics. Bottles were then loosely capped and incubated in the dark at ambient room temperature. Each bottle was subsampled weekly until the first appearance of NO₂⁻, and then quasi daily until complete conversion of NH₄⁺ to NO₃⁻. Samples for nutrient concentration and NO₃⁻ isotope analysis were frozen immediately upon collection until analysis. Samples for NO₂⁻ isotope analysis were processed within an hour of collection for Experiment 1 (described below), and preserved with 1M sodium hydroxide and stored frozen pending isotope analysis for Experiment 2.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Experiment 1 δ¹⁸O_H₂O (‰)</th>
<th>Experiment 2 δ¹⁸O_H₂O (‰)</th>
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<tr>
<td>1</td>
<td>-6.9</td>
<td>-2.2</td>
</tr>
<tr>
<td>2</td>
<td>-6.6</td>
<td>3.8</td>
</tr>
<tr>
<td>3</td>
<td>-5.5</td>
<td>14.6</td>
</tr>
<tr>
<td>4</td>
<td>-4.2</td>
<td>34.0</td>
</tr>
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2.1 DIN analyses --- Nutrient concentrations (NO₃⁻, NO₂⁻, NH₄⁺) were analyzed using standard protocols adapted for automated measurements on a SmartChem® nutrient analyser, a discrete nutrient auto-analyzer (Unity Scientific, Brookfield, Connecticut). NH₄⁺ concentrations were
determined via the Berthelot reaction, the colorimetric reaction of NH$_4^+$ with alkaline phenol, hypochlorite, and sodium nitroprusside (Zhang et al. 1997). NO$_2^-$ was determined through formation of a reddish purple azo dye produced at a pH of 2.0 to 2.5 by coupling diazotized sulfanilamide with N-(1-naphthyl) ethylenediamine dihydrochloride (NED dihydrochloride). The highly colored azo dye is measured colorimetrically at 550 nm. NO$_3^-$ was determined by reduction to NO$_2^-$ by passage of a sample through an open tubular copperized cadmium reactor. The resulting NO$_3^-$ plus NO$_2^-$ are measured via the same protocol for NO$_2^-$ above. (Zhang et al. 1997). Some NO$_2^-$ and NO$_3^-$ concentrations were measured by chemiluminescent detection on a NO$_x$ analyzer (model T200 Teledyne Advanced Pollution Instrument) following reduction to nitric oxide (NO) in a heated iodine solution for NO$_2^-$ (Garside 1982), and reduction in a vanadium (III) solution for NO$_3^-$ plus NO$_2^-$ (Braman 1989).

2.2 N and O Isotope analysis --- NO$_3^-$ $^{15}$N and $^{18}$O measurements were made with the denitrifier method (Sigman et al. 2001; Casciotti et al. 2002; McIlvin and Casciotti 2011), wherein denitrifying bacteria lacking terminal nitrous oxide reductase (P. aureofaciens ATCC 1398) quantitatively convert NO$_3^-$ and NO$_2^-$ in aqueous samples into N$_2$O gas. Working cultures grown 7-10 days are concentrated 10-fold by centrifugation and then split into 3-mL aliquots in 20 mL headspace vials. Each vial is crimp sealed and purged for at least 5 hours with N$_2$ or He. Samples of NO$_3^-$ are injected into the purged vials to obtain 5, 10, or 20 nmoles using a gas tight syringe. Vials are then incubated in the dark overnight to allow for complete conversion of NO$_3^-$ to N$_2$O. The following day, vials are either frozen for later analysis or extracted, purified, and analyzed on a modified Thermo-Scientific Gas Bench II and Delta V Advantage gas
chromatograph isotope ratio mass spectrometer (IRMS). Samples containing less NO$_2^-$ than corresponding NO$_3^-$ were treated with sulfamic acid to remove NO$_2^-$ and then brought back up to a neutral pH with sodium hydroxide prior to bacterial reduction to N$_2$O (Granger and Sigman 2009). For samples where NO$_2^-$ concentrations exceeded NO$_3^-$, NO$_2^-$ was removed via reduction to NO gas by an addition of 1M ascorbic acid and then continuously purged with He for at least 5 hours (Granger et al. 2006). All samples were measured in duplicate against two standards of known isotope composition, IAEA-N3 and USGS-34, which have $\delta^{15}$N and $\delta^{18}$O of 4.7‰ and 25.6‰, and -1.8‰ and -27.9‰, respectively (Gonfiantini et al. 1995; Böhlke et al. 2003). Volume of samples was matched by volume of standards to minimize matrix effects on NO$_3^-$ $\delta^{18}$O (Weigand et al. 2016).

NO$_2^-$ $\delta^{15}$N and $\delta^{18}$O isotope analyses with the azide method (McIlvin and Altabet 2005), wherein NO$_2^-$ is completely reduced to N$_2$O by a 1M sodium azide in a 1.7M acetic acid solution. Samples were diluted in Milli-Q water to 2 mL of 5 µM NO$_2^-$ in 20 mL headspace vials, equivalent to 10 nmoles of NO$_2^-$ in each vial. 2mL of sample, with no dilution, were used when NO$_2^-$ concentrations were lower than 5 µM. All sample vials were flushed with He for 5 minutes, followed by a 15-minute flushing of the sodium azide, acetic acid solution. After flushing, 67 µL of the azide solution was injected into each sample vial using a gas tight syringe and shaken. Samples sat for 30 minutes to allow for complete conversion of NO$_2^-$ to N$_2$O, after which 67 µL of 6M sodium hydroxide were added to terminate the reaction. NO$_2^-$ isotope analyses for Experiment 2 were performed following a slight modification in which the sodium azide, acetic acid solution was buffered with 0.3 M sodium acetate to ensure that samples and standards reacted at the same pH, which affects oxygen atom equilibration. N$_2$O isotope ratios were then
analyzed on the IRMS, as described above. Samples were analyzed in duplicate in each of 2-3 respective batch analyses against a combination of six standards of known isotopic values, WILIS 10, WILIS 11, and WILIS 20, which have δ¹⁵N and δ¹⁸O of -1.7‰ and 13.2‰, 57.1‰ and 8.6‰, and -7.8‰ and 47‰, respectively (Scott Wankel, personal communication); and N-23, N-7373, N-12019, which have δ¹⁵N and δ¹⁸O values of 3.7‰ and 11.4‰, -79.6‰ and 4.5‰, and 2.8‰ and 88.5‰, respectively (Böhlke et al. 2007). Oxygen isotope exchange during the azide reaction was corrected for in Experiment 2 samples by diluting select standards and samples in δ¹⁸O H₂O-enriched water (41.6 ‰) to match those of some samples.

2.3 Regression Analysis --- Type II regressions were conducted in Matlab (Mathworks; Edward Pelter, MBARI) following the method of York et al (1966, 2004), which account for errors in both the X and Y coordinates, in order to derive the slopes and intercepts of δ¹⁸O NO₂ vs. δ¹⁸O H₂O data and δ¹⁸O NO₃,produced vs. δ¹⁸O H₂O data, and ultimately, isotope effect and exchange terms (see discussion section).

3. Results

3.1 Time-dependent evolution of DIN---

3.1.1. The water collected for Experiment 1 posted NO₃⁻ and NH₄⁺ concentrations of 35 µM and 8 µM, respectively, such that initial NH₄⁺ concentrations in experimental incubations were ~58 µM following NH₄⁺ additions on day 1 (Fig.3A). Initial NO₂⁻ concentrations were on the order of 0.2 µM. In all experimental bottles, concentrations of NH₄⁺ increased by 0-9 µM over the first week, suggesting ammonification of dissolved organic nitrogen (DON). Despite potential
ammonification, \( \text{NH}_4^+ \) was completely oxidized to \( \text{NO}_2^- \) by day 14 in all experimental bottles, at which point accumulated \( \text{NO}_2^- \) was on the order of 50–53 µM. \( \text{NO}_2^- \) oxidation to \( \text{NO}_3^- \) occurred concurrently, the onset of which is unclear given coarse time resolution. Accumulated \( \text{NO}_3^- \) ranged between 0–17 µM by day 14 (corresponding to 33–50 µM total \( \text{NO}_3^- \)). \( \text{NO}_2^- \) was completely oxidized to \( \text{NO}_3^- \) by day 21, at which point \( \text{NO}_3^- \) concentrations were on the order of 96-100 µM among treatments, such that \(~59-65\) µM \( \text{NO}_3^- \) was produced throughout the incubation – on par with initial \([\text{NH}_4^+]\). Although some \( \text{NH}_4^+ \) production from the ammonification of DON may have occurred following the onset of \( \text{NH}_4^+ \) oxidation, DIN species showed apparent mass balance at all sampling points, and \( \text{NH}_4^+ \) was recovered quantitatively as \( \text{NO}_3^- \) by the end of the experiment (Fig. 3A) – suggesting that ammonification was only substantive at the onset of the experiment.

![Figure 3. Time course of \( \text{NH}_4^+ \), \( \text{NO}_2^- \), and \( \text{NO}_3^- \) concentrations for all incubations following \( \text{NH}_4\text{Cl} \) addition in (A) Experiment 1 and (B) Experiment 2.](image-url)
3.1.2. The stream water collected for Experiment 2 had lower initial concentrations of NO$_3^-$ and NH$_4^+$, of 1.4 µM and 4.8 µM, respectively, and no detectable NO$_2^-$. Following NH$_4^+$ addition, initial experimental concentrations were thus ~62 µM (Fig. 3B). As with Experiment 1, [NH$_4^+$] increased by 7-10 µM in all bottles over the first ten days of incubation, suggesting ammonification of DON. NH$_4^+$ concentrations remained thus elevated until day 10, after which NH$_4^+$ concentrations decreased to a minimum of ~2.5 µM by day 23. NH$_4^+$ concentrations subsequently remained at ~2.5 µM for 10 days before decreasing to below detection on day 33. NO$_2^-$ production was first detected on day 14, accumulating rapidly to 67-75 µM by day 23, then appeared to increase modestly to concentrations of 72-78 µM among treatments by day 33, although this apparent increase is within the uncertainty of the NO$_2^-$ concentration measurements. Nevertheless, among most experimental bottles, peak [NO$_2^-$] at day 33 exceeded peak [NH$_4^+$] by an average of 2.1 µM, suggesting that ammonification of DON may have occurred concurrently with NH$_4^+$ oxidation. NO$_3^-$ production was first detected on day 38 in a single experimental bottle from treatment 2 that reached 75 µM NO$_3^-$ by day 46. Among other experimental bottles, NO$_3^-$ production began in and around day 43, reaching respective maxima at 71-80 µM NO$_3^-$ between days 53 and 64. Final produced NO$_3^-$ concentrations ranged between 70 µM and 79 µM, thus ~14 µM in excess of initial NH$_4^+$, consistent with the ammonification of DON.

3.2 Evolution of $\delta^{18}O$ and $\delta^{15}N$ of NO$_2^-$ and NO$_3^-$

3.2.1. Experiment 1
3.2.1(a) $\delta^{18}O$ of NO$_2$ and NO$_3^-$ in Experiment 1--- The $\delta^{18}$O$_{NO2}$ in Experiment 1 was first measured on day 8, when [NO$_2^-$] was $\sim$1.9 µM among treatments, corresponding to an $f_{NO2}$ value of 4% - where $f_{NO2}$ is the fraction of the [NO$_2^-$] measured relative the maximum [NO$_2^-$] observed in each incubation, [NO$_2^-$]/[NO$_2^-$max] (Fig. 4A). This metric rests on the premise that [NO$_2^-$max] reflects the sum product of NH$_4^+$ oxidation, not yet diminished by NO$_2^-$ oxidation to NO$_3^-$, an assumption that is not entirely accurate given that up to 10 µM NO$_3^-$ was already produced at peak NO$_2^-$.

Nevertheless, $\delta^{18}$O$_{NO2}$ values first measured on day 8 were -0.7 ± 0.3‰, -1.3 ± 0.4‰, 1.6 ± 0.2‰, and 6.7 ± 0.6‰ for treatments 1-4, respectively, increasing to values of 5.9 ± 0.5‰, 5.3 ± 0.3‰, 6.2 ± 0.2‰, and 6.8 ± 0.3‰ just after peak [NO$_2^-$] on day 15 ($f_{NO2} = 1$). Given coarse time resolution, we take these values to correspond roughly to the “final” $\delta^{18}$O$_{NO2}$ produced from NH$_4^+$ oxidation prior to the onset of NO$_2^-$ oxidation. These final $\delta^{18}$O$_{NO2}$ values were distinctly lower than expected from the weighted source attribution model of Eq. 1, by ~4‰ for treatments 1 and 2 and ~2‰ for treatments 3 and 4 (Fig. 4A). Following peak [NO$_2^-$], $\delta^{18}$O$_{NO2}$ values then decreased by 0-1.5‰ by day 16 as NO$_3^-$ accumulated ($f_{NO2} \sim 0.92$). At the subsequent sampling on day 21, remaining NO$_2^-$ concentrations were too deplete to measure $\delta^{18}$O$_{NO2}$ (~0.2 µM).

The initial $\delta^{18}$O$_{NO3}$ in the stream water was 4.1 ± 0.2‰ ($f_{NO3} \sim 0$; where $f_{NO3} = [NO_3^-]/[NO_3^-max]$) (Fig. 4B). By the first sampling of accumulated NO$_3^-$ on day 14, [NO$_3^-$] had increased by ~15 µM ($f_{NO3} \sim 0.25$) among treatments, although cumulative $\delta^{18}$O$_{NO3, total}$ values remained close to initial values at ~4‰. As [NO$_3^-$] increased further, $\delta^{18}$O$_{NO3}$ values then decreased, resulting in respective final $\delta^{18}$O$_{NO3, total}$ ($f_{NO3} = 1.0$) of -2.3 ± 0.1‰, -2.2 ± 0.1‰, -1.6 ± 0.1‰, and -0.8 ± 0.1‰, for treatments 1 through 4.
Values of $\delta^{18}$O$_{\text{NO}_3}$ corresponding to the NO$_3^-$ specifically produced during the experiments ($\delta^{18}$O$_{\text{NO}_3, \text{produced}}$), derived from the weighted difference from total NO$_3^-$, ranged from $0.91 \pm 0.5\%$ to $4.3 \pm 1.6\%$ among treatments on day 14 ($0.07 < f_{\text{NO}_3} < 0.28$; Fig. 4C). As [NO$_3^-$] increased further, the $\delta^{18}$O$_{\text{NO}_3, \text{produced}}$ decreased to cumulative final values of $-5.8 \pm 0.2\%$, $-5.7 \pm 0.2\%$, $-4.7 \pm 0.3\%$, and $-3.4 \pm 0.3\%$ for treatments 1 through 4, respectively, in apparent proportion to corresponding $\delta^{18}$O$_{\text{H}_2\text{O}}$ treatments. As with $\delta^{18}$O$_{\text{NO}_2}$, however, values of $\delta^{18}$O$_{\text{NO}_3, \text{produced}}$ at the final sampling were substantially lower than expected from fractional source attribution of O atoms (Eq. 2) by 9.3‰, 9.4‰, 9.1‰, and 8.7‰ for respective $\delta^{18}$O$_{\text{H}_2\text{O}}$ treatments (Fig. 4C).

3.2.1(b) $\delta^{15}$N of NO$_2$ and NO$_3^-$ in Experiment 1 --- The corresponding $\delta^{15}$N$_{\text{NO}_2}$ followed a unified trend among treatments in Experiment 1, with values of $-25.0 \pm 0.9\%$ on day 8, increasing by 34‰ to a maximum of $8.9 \pm 0.2\%$ on day 15 ($f_{\text{NO}_2} = 1.0$), (Fig 4D). As with $\delta^{18}$O$_{\text{NO}_2}$, $\delta^{15}$N$_{\text{NO}_2}$ values then decreased following peak [NO$_2^-$], by $\sim$1‰ in all treatments by day 17 ($f_{\text{NO}_2} = 0.92$). Concurrently, values of $\delta^{15}$N$_{\text{NO}_3, \text{total}}$ deceased slightly over the course of the experiment among all treatments (Fig. 4E); Initial values in stream water were $8.2 \pm 0.1\%$ ($f_{\text{NO}_3} = 0$), compared to $7.7 \pm 0.1\%$ ($f_{\text{NO}_3} = 1.0$) at the final sampling. Derived values for the NO$_3^-$ produced cumulatively, $\delta^{15}$N$_{\text{NO}_3, \text{produced}}$, ranged from $7.6 \pm 0.5\%$ to $9.5 \pm 0.5\%$ on day 14 ($0.07 < f_{\text{NO}_3} < 0.28$), then decreased to a cumulative final value of $7.4 \pm 0.2\%$ ($f_{\text{NO}_3} = 1.0$) among all treatments (Fig. 4F). The limited change in $\delta^{15}$N$_{\text{NO}_3, \text{total}}$ throughout the course of the experiment is thus explained by a $\delta^{15}$N of initial NH$_4^+$ pool ($\sim$6.8‰) that was similar to that of initial NO$_3^-$ $\delta^{15}$N (8.2‰) in the incubations.
3.2.2. Experiment 2

3.2.2(a) $\delta^{18}O$ of NO$_2^-$ and NO$_3^-$ Experiment 2 --- $\delta^{18}$O$_{NO2}$ in Experiment 2 was first measured on day 17, when 10-17 µM NO$_2^-$ had accumulated among experimental bottles ($f_{NO2} \sim 0.15$; Fig.4G) posting values of -6.2 ± 0.2‰, -3.2 ± 0.4‰, 5.2 ± 0.4‰, and 18.9 ± 0.5‰ in treatments 1-4, respectively. Values in all incubations then increased by ~8‰ as [NO$_2^-$] increased concurrently by less than 10 µM, $\delta^{18}$O$_{NO2}$ values increased by 2.5‰, 4.3‰, 5.5‰, and 8.9‰ in respective treatments 1-4, resulting in $\delta^{18}$O$_{NO2}$ at [NO$_2^-$] maxima of 3.9 ± 0.4‰, 9.2 ± 0.2‰, 17.8 ± 0.7‰, and 34.4 ± 0.9‰. We consider these latter values to be the final $\delta^{18}$O$_{NO2}$ produced from NH$_4^+$ oxidation. These are notably lower than predicted based on fractional source attribution for O atoms (Eq. 1) for treatments 1, 2, and 3 by 7.1‰, 4.8‰, and 2.0‰ respectively, whereas they are greater than predicted for treatment 4 by 5.2‰. Following the onset of NO$_3^-$ production, $\delta^{18}$O$_{NO2}$ continued to increase slightly to maximum values of 5.1 ± 0.4‰, 10.3 ± 0.1‰, 19.5 ± 0.3‰, and 36.5 ± 0.4‰. After approximately half of the NO$_2^-$ had been oxidized to NO$_3^-$ the $\delta^{18}$O$_{NO2}$ began to decrease, resulting final measurements of $\delta^{18}$O$_{NO2}$ that were 3-7‰ lower than respective maxima.

$\delta^{18}$O$_{NO3, total}$ values rapidly increased at the onset of NO$_3^-$ production, from an initial ($f_{NO3} = 0.0$) $\delta^{18}$O$_{NO3}$ value of -5.5 ± 0.8‰ in stream water, to 4.1 ± 1.0‰, 7.6 ± 0.3‰, 15.0 ± 0.5‰, and 30.4 ± 0.7‰ (0.05 < $f_{NO3} < 0.15$) in respective treatments 1-4 (Fig. 4H), reflecting mixing of newly produced NO$_3^-$ with the modest ambient pool of stream water NO$_3^-$ (of 1.4 µM). $\delta^{18}$O$_{NO3, total}$ values then evolved gradually as NO$_2^-$ oxidation proceeded, posting final ($f_{NO3} = 1.0$)
\( \delta^{18}O_{\text{NO}_3,\text{total}} \) values of 0.1 ± 0.1‰, 5.1 ± 0.3‰, 14.7 ± 0.7‰, and 32.9 ± 0.4‰. The corresponding \( \delta^{18}O_{\text{NO}_3,\text{produced}} \) produced at the onset of NO\(_2^-\) oxidation (0.05 < \( f_{\text{NO}_3} \) < 0.19) were 3.8 ± 0.7‰, 11.4 ± 0.6‰, 18.6 ± 0.7‰, and 37.7 ± 0.7‰, for treatments 1-4, respectively (Fig. 4I), gradually decreasing among treatments to final values (\( f_{\text{NO}_3} = 1 \)) of 0.0 ± 0.1‰, 5.3 ± 0.2‰, 15.1 ± 0.8‰, and 33.6 ± 0.4‰. As such, the final \( \delta^{18}O_{\text{NO}_3,\text{produced}} \) values were lower than would be predicted by Eq. 2 for treatments 1-3 by 6.6‰, 5.3‰, and 2.0‰, respectively, and higher than predicted by 3.2‰ for treatment 4.

3.2.2(b) \( \delta^{15}N \) of NO\(_2^-\) and NO\(_3^-\) Experiment 2 --- The \( \delta^{15}N \) of the NO\(_2^-\) produced initially (0.05 < \( f_{\text{NO}_2} < 0.11 \); Fig. 4J) was -18.6 ± 0.4‰, averaged among treatments, and increased by ~29‰ to a maximum value 9.8 ± 0.2‰ at peak NO\(_2^-\) concentrations (\( f_{\text{NO}_2} = 1.0 \)). As NO\(_2^-\) was depleted, \( \delta^{15}N_{\text{NO}_2} \) decreased to ~ -6.0‰ (\( f_{\text{NO}_2} \sim 0.10 \)). The stream water NO\(_3^-\) had an initial \( \delta^{15}N_{\text{NO}_3} \) value of 10.8 ± 0.2‰. In a similar trend to \( \delta^{18}O_{\text{NO}_3,\text{total}} \), \( \delta^{15}N_{\text{NO}_3,\text{total}} \) values rapidly increased at the onset of NO\(_2^-\) oxidation, rising to an average value of 16.2 ± 0.9‰ (\( f \sim 0.10 \)) among treatments, before gradually decreasing to a final value of 7.8 ± 0.1‰ (Fig. 4K). The \( \delta^{15}N_{\text{NO}_3,\text{produced}} \) values at the onset of NO\(_3^-\) production (\( f_{\text{NO}_3} < 0.10 \)) ranged from 15.8 ± 0.4‰ to 20.0 ± 0.6‰ (Fig. 4L). The \( \delta^{15}N_{\text{NO}_3,\text{produced}} \) decreased gradually as NO\(_3^-\) was produced, to a cumulative final \( \delta^{15}N_{\text{NO}_3,\text{produced}} \) (\( f = 1 \)) of 7.7 ± 0.1‰ among treatments.
Figure 4. Concentration-dependent evolution of $\delta^{18}$O$_{\text{NO2}}$ (A), $\delta^{15}$O$_{\text{NO3\ total}}$ (B), $\delta^{15}$O$_{\text{NO3\ produced}}$ (C), $\delta^{15}$N$_{\text{NO2\ max}}$ (D), $\delta^{15}$N$_{\text{NO3\ total}}$ (E), and $\delta^{15}$N$_{\text{NO3\ produced}}$ (F) during Experiment 1; and $\delta^{18}$O$_{\text{NO2}}$ (G), $\delta^{18}$O$_{\text{NO3\ total}}$ (H), $\delta^{15}$O$_{\text{NO3\ produced}}$ (I), $\delta^{15}$N$_{\text{NO2\ max}}$ (J), $\delta^{15}$N$_{\text{NO3\ total}}$ (K), and $\delta^{15}$N$_{\text{NO3\ produced}}$ (L) during Experiment 2. The x-axis, f$_{\text{NO2}}$, corresponds to the fraction of NO$_2$ at the time of sampling relative to the maximum concentration of NO$_2$ observed over the entire experiment. The color and marker scheme is the same as in Fig. 3, with NO$_2$ presented in shades of blue and NO$_3$ presented in shades of orange, and where shading (light to dark) corresponds to respective treatments [1-4]. In addition, diamond markers correspond to treatment 1, circle markers correspond to treatment 2, triangle markers correspond to treatment 3, and square markers correspond to treatment 4. Dashed, colored lines correspond to the expected $\delta^{18}$O of NO$_2$ (panels A and G) and NO$_3$ (panels C and I) for each $\delta^{18}$O$_{\text{NO2}}$ treatment according to Eq. 1 and 2. Error bars are analytical error for all panels with the exception of $\delta^{18}$O and $\delta^{15}$N of NO$_3$ produced (C, F, I, and L), in which case the errors are derived from propagation of analytical uncertainty on $\delta^{18}$O and $\delta^{15}$N of NO$_3$. 

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3.3 Dependence of $\delta^{18}O_{\text{NO}_2}$ and $\delta^{18}O_{\text{NO}_3}$ on $\delta^{18}O_{\text{H}_2\text{O}}$--- The values of $\delta^{18}O_{\text{NO}_2}$ at peak NO$_2^-$ displayed a linear dependence on $\delta^{18}O_{\text{H}_2\text{O}}$ in both experiments (Fig 5A and 5B). Respective slopes of fitted linear regressions for both experiments are $0.63 \pm 0.14$ and $0.73 \pm 0.003$ respectively, greater than the value of $0.50$ otherwise expected from Eq. 1, thus signaling a greater dependence of $\delta^{18}O_{\text{NO}_2}$ on the $\delta^{18}O$ of H$_2$O. The respective intercepts, $9.5 \pm 0.8$ and $5.2 \pm 0.0$, are lower than the value of $12.1$ (one half of $\delta^{18}O_{\text{O}_2}$) expected from Eq. 1, assuming a

![Graphs showing linear relationships between $\delta^{18}O_{\text{NO}_2}$ and $\delta^{18}O_{\text{H}_2\text{O}}$.](image)

*Figure 5. Intermediate $\delta^{18}O_{\text{NO}_2}$ vs. $\delta^{18}O_{\text{H}_2\text{O}}$ for Exp. 1 (A) and Exp. 2 (B) and final $\delta^{18}O_{\text{NO}_3}$ produced vs. $\delta^{18}O_{\text{H}_2\text{O}}$ for Exp. 1 (C) and Exp. 2 (D). Dashed lines and equations are linear regressions fit to the data using Matlab. $\delta^{18}O_{\text{NO}_2}$ and $\delta^{18}O_{\text{NO}_3}$ expected from Eq. 1 and 2 are represented in each plot in grey. Error bars are the standard deviation of triplicate bottle measurements.*
\( \delta^{18}O_{O_2} \) of 24.2\% for air-equilibrated \( O_2 \) (Kroopnick & Craig, 1972). We note that given the very slow growth rates of nitrifiers in our incubations as well as agitation during sampling, \( \delta^{18}O \) of \( O_2 \) was likely close to equilibrium value throughout the experiments.

Similarly, \( \delta^{18}O \) of final produced \( NO_3^- \) also show linear dependence, with slopes of 0.91 ± 0.04 and 0.93 ± 0.01 and intercepts of 0.4 ± 0.3 and 2.0 ± 0.1 (Fig 5C and 5D). As with \( \delta^{18}O_{NO_2} \), the regression slopes are greater than the expected 0.66 and the intercepts lower than the 8.1 expected from the fractional source contribution model, Eq. 2.

4. Discussion

The oxygen isotope composition of \( NO_2^- \) and \( NO_3^- \) produced from the oxidation of \( NH_4^+ \) showed a direct dependence on \( \delta^{18}O_{H_2O} \) among treatments, albeit, in proportions different than expected from simple fractional contributions of O atom sources. The divergence from direct source attribution suggests the influence of kinetic isotope effects on O during incorporation, as well as O atom equilibration of \( NO_2^- \) with \( H_2O \) and associated equilibrium isotope effects, as documented in cultures of nitrifying prokaryotes (Buchwald & Casciotti, 2010; Buchwald et al., 2012; Casciotti et al., 2010; Snider et al., 2010). Below, we first examine the evolution of \( NO_2^- \) O isotopes to discern the potential influence of isotope effects and O atom equilibration during its production (\( NH_4^+ \) oxidation) and during its consumption (\( NO_2^- \) oxidation). We then assess the concurrent evolution of \( NO_3^- \) isotopes to confirm inferences gleaned from \( NO_2^- \) isotope measurements. Based on the dynamics uncovered, we discuss implications for detecting nitrification in the environment from \( NO_3^- \) \( \delta^{18}O \), and for the interpretation of \( NO_3^- \) isotope distributions in freshwater environments.
4.1 $\delta^{18}O$ of NH$_4^+$ oxidation ---

On the basis that the O atoms incorporated during the oxidation of NH$_4^+$ to NO$_2^-$ derive from molecular O$_2$ and H$_2$O (Andersson & Hooper, 1983), then the $\delta^{18}O$ of NO$_2^-$ produced by nitrification in our experiments would correspond to the weighted sum of the $\delta^{18}O$ of end members (Eq. 1), barring the influence of incorporation isotope effects. The $\delta^{18}O$ of NO$_2^-$ thus produced would also remain invariant throughout the time course of the experiments – as the $\delta^{18}O$ of the O$_2$ and H$_2$O substrate pools remain constant – unless NO$_2^-$ was subject to progressive O-atom equilibration with H$_2$O. Contrary to these expectations, however, the $\delta^{18}O$ of NO$_2^-$ produced at the onset of NH$_4^+$ oxidation in both experiments was substantially lower than predicted by the weighted sum (Eq. 1; Fig. 4A and 4G). The low $\delta^{18}O$ of NO$_2^-$ values initially produced suggest isotopic discrimination during O-atom incorporation from either O$_2$, H$_2$O, or both. As NH$_4^+$ oxidation proceeded, the $\delta^{18}O$ of NO$_2^-$ increased to respective $\delta^{18}O$ maxima at peak [NO$_2^-$] in all but one treatment, which had the highest experimental $\delta^{18}O_{H2O}$ (Experiment 2), in which $\delta^{18}O_{NO2}$ decreased. This $\delta^{18}O_{NO2}$ change among treatments is consistent with NO$_2^-$ equilibration with H$_2$O and associated isotope effect, where $\delta^{18}O_{NO2}$ equilibrates to a value on the order of $\delta^{18}O_{H2O} + 13\%$ at room temperature (Buchwald & Casciotti, 2013; Casciotti et al., 2007). At peak NO$_2^-$ concentrations in both experiments, the $\delta^{18}O_{NO2}$ was lower than expected for full equilibration among treatments, suggesting partial equilibration.

The observations may thus show better correspondence to a more comprehensive model of NH$_4^+$ oxidation that considers incorporation isotope effects on oxygen atoms of molecular O$_2$ and H$_2$O, as well as fractional oxygen atom equilibration of NO$_2^-$ with H$_2$O with a corresponding equilibrium isotope effect (Eq. 3; Casciotti et al., 2010; Buchwald et. al, 2012).
Where $X_{\text{NO}_2,1}$ is the fraction of NO$_2^-$ oxygen atoms that have equilibrated with H$_2$O, $^{18}_{\text{k,O}_2}$ is the kinetic isotope effect for O$_2$ incorporation, $^{18}_{\text{k,H}_2\text{O},1}$ is the kinetic isotope effect for H$_2$O incorporation, and $^{18}_{\text{eq}}$ is the equilibrium isotope effect for NO$_2^-$ equilibration with water (Casciotti et al. 2010).

$$\delta^{18}\text{O}_{\text{NO}_2} = \left[\frac{1}{2} (\delta^{18}\text{O}_{\text{O}_2} - ^{18}_{\text{k,O}_2}) + \frac{1}{2} (\delta^{18}\text{O}_{\text{H}_2\text{O}} - ^{18}_{\text{k,H}_2\text{O},1}) \right] (1 - X_{\text{NO}_2,1}) + (\delta^{18}\text{O}_{\text{H}_2\text{O}} + ^{18}_{\text{eq}})(X_{\text{NO}_2,1}) \quad (3)$$

Equation 3 can be re-arranged to a linear model describing $\delta^{18}\text{O}_{\text{NO}_2}$ vs. $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ (Fig. 7A and B).

$$\delta^{18}\text{O}_{\text{NO}_2} = \left(\frac{1}{2} + \frac{1}{2} X_{\text{NO}_2,1}\right) \delta^{18}\text{O}_{\text{H}_2\text{O}} \quad (4)
+ \frac{1}{2} \left[ (\delta^{18}\text{O}_{\text{O}_2} - ^{18}_{\text{k,O}_2} - ^{18}_{\text{k,H}_2\text{O},1})(1-X_{\text{NO}_2,1}) \right] + (X_{\text{NO}_2,1}^{18}_{\text{eq}})$$

From Eq. 4 and the linear regression of peak NO$_2^-$ $\delta^{18}\text{O}_{\text{NO}_2}$ (Fig. 5A and 5B), we can estimate the fraction of NO$_2^-$ O atoms that had exchanged with H$_2$O by the time of sampling ($X_{\text{NO}_2,1}$) from the regression slope and the combined O incorporation isotope effect ($^{18}_{\text{k,O}_2} + ^{18}_{\text{k,H}_2\text{O},1}$) during NH$_4^+$ oxidation from the regression intercept. We assume that O$_2$ in our experimental bottles was in equilibrium with air, $\delta^{18}\text{O}_{\text{O}_2}$ of 24.2‰ (Kroopnick & Craig, 1972), and an NO$_2^-$H$_2$O equilibrium isotope effect ($^{18}_{\text{eq}}$) of 13‰ for both abiotic and biologically mediated exchange (Buchwald & Casciotti, 2013; Karen L. Casciotti et al., 2007). From the slope of the $\delta^{18}\text{O}_{\text{NO}_2}$ regression (Fig. 5A and 5B) we derive $X_{\text{NO}_2,1}$ values of 26 ± 27% for Experiment 1 and 47 ± 1% for Experiment 2. The sizeable uncertainty in the estimates for Experiment 1 derives from the poor sampling resolution, and may also reflect that some fraction of NO$_2^-$ was already oxidized to NO$_3^-$ at peak [NO$_2^-$]. Nevertheless, the proportion of NO$_2^-$ O atom
equilibrated with H₂O computed here are comparable to values observed in a NH₄⁺ oxidizing cultures (Casciotti et al., 2010; Table 2).

We note that our equilibration estimates here conflate both abiotic and biologically-mediated equilibration, contrasting estimates from cultures in Table 2 which represent fraction of biologically-mediated exchange exclusively. O atom equilibration between NO₂⁻ and H₂O occurs inorganically, as a function of pH, temperature, and NO₂⁻ residence time, but can also be facilitated by some NH₄⁺ oxidizing bacteria and archaea (Casciotti et al., 2010). Inorganic equilibration is expectedly slow at the pH of our incubations, on order of 10 - 30% over 3 weeks (Casciotti et al., 2007).

While we cannot readily distinguish between abiotic and biologically mediated exchange in our incubations, we observed a relatively rapid increase in ¹⁸O(NO₂) during the first 3-9 days of active NH₄⁺ oxidation in Experiment 2 (Fig. 6), which we interpret as dominated by biotically-mediated exchange during active NH₄⁺ oxidation. Near the end of the steep increase in ¹⁸O(NO₂), on day 23, the fraction of NO₂⁻ isotopically equilibrated with water was ~ 27%, in the upper range of biologically mediated exchange observed for NH₄⁺ oxidizing cultures (Casciotti et al., 2010; Table 2). Equilibration was subsequently slower during the following two weeks where

Figure 6. Time dependent evolution of ³⁰⁸O(NO₂) produced among treatments and bottle triplicates in Experiment 2. Error bars are standard deviations of analytical replicates.
ambient NO$_2^-$ remained elevated (≥70 µM; Fig. 6), reaching 45% prior to the onset of NO$_3^-$ production. The extent to which this slower equilibration was abiotic or biotically catalyzed is uncertain. Equilibration on the accumulated NO$_2^-$ pool presumably continued during subsequent NO$_3^-$ production, although apparent inverse isotopic discrimination of NO$_2^-$ O atoms at the onset of NO$_3^-$ production obfuscates any visual trend of equilibration in the δ$^{18}$O$_{NO2}$ data (Fig. 6).

From the intercepts of the δ$^{18}$O$_{NO2}$ vs. δ$^{18}$O$_{H2O}$ regressions (Fig. 5A and 5B), we derive combined O atom incorporation isotope effects, $\delta^{18}_k,_{O2} + \delta^{18}_k,_{H2O,1}$, of 7.6 ± 17.8‰ and 27.5 ± 4.0‰ for Experiments 1 and 2 respectively (Eq. 6). As mentioned in the Results, some NO$_2^-$ had already been consumed at the sampling for intermediate NO$_2^-$ used in Fig. 5A, rendering uncertain the estimate of the combined isotope effect for Experiment 1. Nevertheless, values for both experiments are within the observed range of culture and seawater incubation studies.

| Table 2. Isotope effects and fraction of NO$_2^-$ isotopically equilibrated with H$_2$O (X$_{NO2}$) in our experiments, compared to existing estimates from nitrifier culture studies and seawater incubations ([1] Buchwald et al., 2012; [2] Casciotti et al., 2010; [3] Casciotti et al., 2003; [4] Mariotti et al., 1981; [5] Buchwald and Casciotti, 2010). X$_{NO2}$ values from culture experiments correspond to biologically mediated equilibration only, whereas X$_{NO2}$ values from our experiments and seawater incubations conflate abiotic and biologically mediated equilibration. *Kinetic isotope effect does not factor out competing contribution of coincident O-atom equilibration with NO$_2^-$.

|                | Exp. 1 (n = 1) | Exp. 2 (n = 1) | Seawater Incubations (n = 7)$^1$ | Co-cultures (n = 6)$^1$ | NH$_4^+$ oxidizing cultures (n = 10)$^{2,3,4}$ | NO$_2^-$ oxidizing cultures (n = 3)$^5$
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<td>X$_{NO2,1}$ (%)</td>
<td>26 ± 27</td>
<td>47 ± 1</td>
<td>35 – 100</td>
<td>16 – 28</td>
<td>1 – 25</td>
<td>ND</td>
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<tr>
<td>X$_{NO2,2}$ (%)</td>
<td>74 ± 12</td>
<td>78 ± 3</td>
<td>48 – 100</td>
<td>0 – 26</td>
<td>0 – 3</td>
<td>ND</td>
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<tr>
<td>$^{18}_k,H2O,1+^{18}_k,O2$ (%)</td>
<td>7.6 ± 17.8</td>
<td>27.5 ± 4.0</td>
<td>11 – 20</td>
<td>16 – 23</td>
<td>18 – 30</td>
<td>ND</td>
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<tr>
<td>$^{18}_k,H2O,2$ (%)</td>
<td>22.5 ± 6.5</td>
<td>13.5 ± 2.0</td>
<td>1 – 27</td>
<td>6 – 12</td>
<td>ND</td>
<td>9 – 25</td>
</tr>
<tr>
<td>$^{18}_k,NO2$ (%)</td>
<td>ND</td>
<td>-3.9 ± 0.3‡</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-10 – -1.4</td>
</tr>
<tr>
<td>$^{15}_k,AMO$ (%)</td>
<td>ND</td>
<td>34.5 ± 0.2</td>
<td>ND</td>
<td>ND</td>
<td>14 – 38</td>
<td>ND</td>
</tr>
<tr>
<td>$^{15}_k,NO2$ (%)</td>
<td>ND</td>
<td>-10.3 ± 0.4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-21 – -9</td>
</tr>
</tbody>
</table>
In all, the $\delta^{18}O$ of NO$_2^-$ produced from biological NH$_4^+$ oxidation in stream water incubations was subject to incorporation isotope effects (for either H$_2$O or O$_2$ O atom incorporation, or both) and was further dependent on $\delta^{18}O$ of H$_2$O as a function of NO$_2^-$ equilibration and the associated isotope effect.

### 4.2 $\delta^{18}O$ of NO$_2^-$ oxidation ---

During biological NO$_2^-$ oxidation, the O atom appended to NO$_3^-$ derives from H$_2$O (Kumar et al., 1983). To a first approximation, the $\delta^{18}O$ of NO$_3^-$ produced during nitrification should reflect to the weighted sum of the $\delta^{18}O$ end members (Eq. 2), barring the potential influence of (a) an O atom incorporation isotope effect, (b) isotopic exchange of NO$_2^-$ with H$_2$O, and (c) a kinetic isotope effect on O atoms of NO$_2^-$ during its oxidation to NO$_3^-$. The $\delta^{18}O$ of NO$_3^-$ thus produced would also remain constant within individual treatments throughout the experiment. Countering the latter expectation, the $\delta^{18}O_{NO3, produced}$ at onset of NO$_3^-$ production decreased continually among all treatments in both experiments (Fig. 4C and 4I). This trend is consistent with inverse isotope discrimination on the O atoms during NO$_2^-$ oxidation ($^{18}C_{k,NO2}$), which has been documented in culture studies of NO$_2^-$ oxidizing bacteria (Buchwald & Casciotti, 2010; Casciotti, 2009). Inverse isotope fractionation of
O is also apparent in the $\delta^{18}O_{NO_2}$ data from the decreasing trend in $\delta^{18}O_{NO_2}$ as it is oxidized to NO$_3^-$: The high temporal resolution in Experiment 2 allows for an estimate of the inverse O isotope effect on NO$_2^-$ from the closed-system Rayleigh product equation (Mariotti et al., 1981):

$$\delta_{product} = \delta_{substrate,initial} - \varepsilon \times \frac{\ln(f)}{(1-f)} \tag{5}$$

where $f$ is fraction of the substrate (NO$_2^-$) remaining relative to the initial substrate concentration. The resulting value of $^{18}\varepsilon_{k,NO2}$ is $\sim$ -3.9‰ (Fig. 7), which is comparable to values reported for culture studies (Buchwald and Casciotti, 2010; Table 2). However, we note that this value is uncertain given a potentially competing effect of ongoing NO$_2^-$ equilibration.

In order to determine whether NO$_2^-$ was subject to further O atom equilibration following the onset of NO$_3^-$ production, we investigate the correspondence of NO$_3^-$ O isotope dynamics to the more comprehensive description of NO$_2^-$ oxidation that account for equilibration as well as potential incorporation isotope effects (Eq. 6; Buchwald and Casciotti, 2010; Buchwald et. al, 2012). The potential for equilibration of NO$_2^-$ with H$_2$O during NO$_2^-$ oxidation is also featured in Eq. 6. However, the biological catalysis of equilibration was found to be negligible during NO$_2^-$ oxidation (Buchwald and Casciotti, 2010). Nevertheless Eq. 6 accounts for pH-dependent inorganic equilibration, which occurs on pertinent time scales and is denoted as $X_{NO2}$ for completeness. We note that as presented, Eq. 6 neglects the inverse kinetic isotope effect for NO$_2^-$ conversion to NO$_3^-$ given complete oxidation of NO$_2^-$ to NO$_3^-$; it would however be pertinent in the case of incomplete NO$_2^-$ oxidation.

$$\delta^{18}O_{NO3, nitriified} = \frac{2}{3} [(1 - X_{NO2})\delta^{18}O_{NO2, initial} + X_{NO2}(\delta^{18}O_{H2O} + 18\varepsilon_{eq})] + \frac{1}{3}(\delta^{18}O_{H2O} - 18\varepsilon_{k,H2O,2}) \tag{6}$$
With the substitution of $\delta^{18}O_{NO_2}$ terms from Eq. 3, Eq. 6 is rearranged to conform to a linear formulation of $\delta^{18}O_{NO_3, nitrified}$ vs. $\delta^{18}O_{H_2O}$. From the linearized Eq. 7, we estimate the final fraction of NO$_2$- O atoms exchanged with H$_2$O over the entire time course of our experiments ($X_{NO_2,2}$) from the slope, and the third O incorporation isotope effect ($^{18}\varepsilon_{k,H_2O,2}$) during NO$_2^-$ oxidation from the intercept (Buchwald and Casciotti, 2010; Buchwald et. al, 2012). To solve for $X_{NO_2,2}$ and $^{18}\varepsilon_{k,H_2O,2}$ in Eq. 7, we assume the same values for $\delta^{18}O_{O_2}$ and $^{18}\varepsilon_{eq}$ as for $\delta^{18}O_{NO_2}$ analysis and we use the respective $^{18}\varepsilon_{k,O_2} + ^{18}\varepsilon_{k,H_2O,1}$ derived from each experiment.

\[
\delta^{18}O_{NO_3, nitrified} = \left( \frac{2}{3} + \frac{1}{3}X_{NO_2,2} \right)\delta^{18}O_{H_2O} \\
\quad + \frac{1}{3} \left[ (\delta^{18}O_{O_2} - ^{18}\varepsilon_{k,O_2} - ^{18}\varepsilon_{k,H_2O,1})(1-X_{NO_2,2}) - ^{18}\varepsilon_{k,H_2O,2} \right] + \frac{2}{3} \left( X_{NO_2,2}^{18}\varepsilon_{eq} \right)
\]  

The total fraction of NO$_2^-$ O-atom equilibration with H$_2$O was 74 $\pm$ 12% and 78 $\pm$ 3% in Experiments 1 and 2 respectively. These values are comparable to those observed during incubations of natural seawater assemblages (Buchwald et al., 2012) (Table 2), where high concentrations (upwards of 50 µM) of accumulated NO$_2^-$ remained present for at least 13 days prior to the onset of NO$_2^-$ oxidation. In similar experiments with NH$_4^+$ oxidizing and NO$_2^-$ oxidizing co-cultures (Buchwald et al., 2012), less than 28% of NO$_2^-$ O atoms had exchanged with H$_2$O. This difference is presumably due to substantially shorter residence times of NO$_2^-$ in the co-culture experiments, resulting in a decreased potential for abiotic equilibration. Biologically-mediated exchange by NO$_2^-$ oxidizing cultures is thought to be negligible (Buchwald and Casciotti, 2010). Therefore, the increase of fraction of NO$_2^-$ exchanged calculated from
\(\delta^{18}O_{NO_2}\) data to 74\% and 78\% (from values of 26\% and 47\% at the onset of \(NO_3^-\) production) may derive dominantly from abiotic equilibration.

From the intercepts of \(\delta^{18}O_{NO_3,\text{produced}}\) vs. \(\delta^{18}O_{H_2O}\) linear regressions, we derive respective values of 22.5 ± 6.5‰ and 13.5 ± 2.0‰ for the O atom incorporation isotope effect during \(NO_2^-\) oxidation in Experiments 1 and 2. These values are on par with those observed in \(NO_2^-\) oxidizing monocultures, and incubations of nitrifying co-culture and natural assemblage seawater experiments (Buchwald & Casciotti, 2010; Buchwald et al., 2012). Thus, the \(NO_2^-\) oxidation step in stream water incubations is subject to both incorporation and inverse kinetic isotope effects, as well as ongoing \(NO_2^-\) equilibration, analogous to cultures and marine incubations (Table 2).

4.3 N isotope dynamics ---

While not the focus of this study, the N isotope dynamics of experimental incubations provide additional support regarding the influence of isotopic discrimination on produced \(NO_3^-\).

Initial \(NO_2^-\) N was isotopically light and increased during production, which is consistent with a

![Figure 8. Rayleigh fractionation for N isotope in \(NO_2^-\) and \(NO_3^-\): \(\delta^{15}N_{NO_2}\) vs. \(f\ln(f)/(1-f)\) during \(NH_4^+\) oxidation (A), \(\delta^{15}N_{NO_2}\) vs. \(\ln(f)\) during \(NO_2^-\) oxidation (B), and \(\delta^{15}N_{NO_3}\) vs. \(f\ln(f)/(1-f)\) during \(NO_2^-\) oxidation (C). All 12 incubation bottles are represented. Kinetic isotope effects were calculated from the slopes of each line, according to the Rayleigh accumulated product equation for (A) and (B) and the Rayleigh substrate equation for (C). Error bars are analytical errors on individual measurements. Error on the \(\delta^{15}N\) is error on the slope according to Type II linear regression.](image)
kinetic isotope effect on N during NH₄⁺ oxidation. Indeed, a plot of highly resolved δ¹⁵N_NO₂ from Experiment 2 and the Rayleigh product equation (Eq. 5; Fig 8A), yield an isotope effect (ε_k_AMO) of 34.5 ± 0.2‰. This value is relatively high in comparison with marine isolates but is concordant with freshwater bacterial isolates *Nitrosomonas europaea* and *Nitrosomonas eutropha* (Casciotti et al., 2003; Mariotti et al., 1981; Table 2). In turn, both substrate and product equations for a closed system Rayleigh model (Eqs. 5 and 8) provide estimates of kinetic N isotope effect for NO₂⁻ oxidation, where the substrate equation is:

\[
\delta_{\text{substrate}} = \delta_{\text{substrate, initial}} - \varepsilon \times \ln(f)
\]

Where \(f\) in both formulations is defined as \([\text{NO}_2^-]/[\text{NO}_2^-_{\text{max}}]\). Equations 5 and 8 yield estimates of -10.3 ± 0.4‰ and -8.7 ± 0.2‰ (Fig. 8B and 8C), respectively. Although not identical, both values of \(^{15}\varepsilon_{\text{k,NO}_2}\) express inverse isotope fractionation during NO₂⁻ oxidation and are within the range observed in cultures (Buchwald and Casciotti, 2010; Table 2).

4.4 Implications for interpretation of NO₃⁻ isotopes in the environment ---

The observations in this study suggest that nitrification by freshwater community produces NO₃⁻ with an oxygen isotopic composition that reflects not only the source of O atoms, H₂O and O₂, but that it is also sensitive to large O-atom incorporation isotope effects and isotopic equilibration of NO₂⁻ with H₂O. Our results agree with analogous findings in other recent nitrification studies of sea water cultures and natural assemblage incubations (Buchwald et al, 2012) as well as forest soil incubations (Fang et al., 2012; Snider et al., 2010). Our
observed $\delta^{18}$O values of nitrified NO$_3^-$ at naturally occurring $\delta^{18}$O$_{H2O}$ were repeatedly lower than would be expected from just end-member contribution (Eq. 2). Therefore, assuming median values for O incorporation isotope effects, the only way that the $\delta^{18}$O of nitrified NO$_3^-$ could yields estimates as high as predicted by Eq. 2 at naturally occurring $\delta^{18}$O$_{H2O}$ would be if the $\delta^{18}$O of ambient O$_2$ were highly elevated, and if there were little to no NO$_2^-$ equilibration with H$_2$O. Elevated $\delta^{18}$O$_{O2}$ may be possible in highly respired soils with limited input of new O$_2$. Soil bacteria have been shown to respire O$_2$ with an isotope effect on the order of 20‰ (Lane & Dole, 1956). Assuming our values for O-atom incorporation isotope effects and no isotopic equilibration of NO$_2^-$, then $^{18}$O enrichment of O$_2$ from respiration would have to increase the $\delta^{18}$O$_{O2}$ from 24.2‰ (equilibrium with the atmosphere) to ~ 60‰ in order to fortuitously conform to the $\delta^{18}$O$_{NO3}$ expected from Eq. 2 at a $\delta^{18}$O$_{H2O}$ of -5‰. In the event of NO$_2^-$ equilibration, then the $\delta^{18}$O of O$_2$ would have to be even heavier in order to yield $\delta^{18}$O$_{NO3}$ in the range predicted by Eq. 2.

The amount of NO$_2^-$ exchange that may occur during nitrification is dependent on pH, temperature, nitrification rates, and the residence time of NO$_2^-$. The rate of abiotic exchange is pH and temperature dependent, such that rates are faster in more acidic, warmer waters. As in our experiments and other nitrification incubations (Buchwald et al., 2012; Snider et al., 2010) NH$_4^+$ oxidation and NO$_2^-$ oxidation can become decoupled, thus allowing for high concentrations of NO$_2^-$ to accumulate and increasing the amount of exchange that occurs. Snider et al. 2010 observed that the fraction of abiotic exchange in soils is inversely proportional to net nitrification rates, showing that slower rates allowed for more opportunity for exchange, even when NH$_4^+$ oxidation and NO$_2^-$ oxidation were relatively coupled. However,
due to low concentrations and residence times of NO$_2^-$ in the environment, and that significant abiotic equilibration can take weeks to months at near neutral pH (Buchwald & Casciotti, 2013), it is difficult to quantify how much abiotic exchange impacts the $\delta^{18}$O of nitrified NO$_3^-$. Nevertheless, organism-catalyzed exchange seems likely to have a significant impact on the $\delta^{18}$O of nitrified NO$_3^-$. Biologically mediated exchange during NH$_4^+$ oxidation has been found to be up to 26% for pure cultures of NH$_4^+$ oxidizers and closely coupled co-cultures (Buchwald et al., 2012; Karen L. Casciotti et al., 2010). A study of soil incubations found exchange to range from 37% in a temperate forest soil to 52% in a low organic matter agricultural soil with little NO$_2^-$ accumulation (Snider et al., 2010). Interestingly, Snider et al., 2010 observed in one soil type at naturally occurring $\delta^{18}$O$_{H2O}$, the $\delta^{18}$O of NO$_3^-$ produced was similar to, and in some replicates greater than, what would be expected by Eq. 2. This may be possible if NO$_2^-$ was almost fully exchanged and $^{18}e_{k,H2O,2}$, which was not derived in this study, was at the lowest observed value in these particular incubations (~1‰; Buchwald et al., 2012). Conformity to Eq. 2 may be also be explained by potentially co-occurring denitrification, thus increasing the $\delta^{18}$O of ambient NO$_3^-$, as some replicates of this soil type had $\delta^{18}$O$_{NO3}$ greater than predicted by Eq. 2. Nevertheless, most values for $\delta^{18}$O$_{NO3}$ from Snider et al., 2010 were lower than predicted by Eq. 2 by ~7‰ and when $\delta^{18}$O$_{NO3}$ produced was plotted against $\delta^{18}$O$_{H2O}$ (as in Fig. 5), the slopes were greater than 2/3 for all soil types, indicating that more than 2/3 of the O atoms in NO$_3^-$ were derived from H$_2$O. Similarly, Fang et al., 2012 observed a range $\delta^{18}$O NO$_3^-$ produced by nitrification in temperate forest soils of -9.3 – 2.9‰, which were lighter than predicted by 5.2 – 9.5‰. They also saw an increasing trend between $\delta^{18}$O$_{NO3}$ and ground elevation, which they attribute to more NO$_2^-$ exchange at elevation due the higher acidity of soils.
Instances where the $\delta^{18}$O of nitrified NO$_3^-$ is greater than predicted by Eq. 2 (Burns, 2002; Mayer et al., 2001; Spoelstra et al., 2007) have often been reported. For example, Mayer et al., 2001 reported $\delta^{18}$O$_{NO3}$ values for NO$_3^-$ produced nitrification in acid forest floors to be up to 12‰ greater than predicted with a $\delta^{18}$O$_{H2O}$ of -8‰. The authors attributed the elevated $\delta^{18}$O$_{NO3,nitrified}$ value to the potential isotopic enrichment of O$_2$ from respiration and the possibility that “heterotrophic nitrification” imparts a high $\delta^{18}$O on nitrified nitrate. While there is no a priori expectation that the reactions and catalyzing enzymes for nitrification by heterotrophs should differ from that by autotrophs, organic nitrogen compounds reportedly provide O atoms to NO$_3^-$ during heterotrophic nitrification (Doxtrader, 1965; Focht and Verstraete, 1977; Wood 1988; Wood 1990), thereby eliminating any dependence of the $\delta^{18}$O of NO$_3^-$ on O$_2$, and possibly H$_2$O. Denitrification occurring simultaneously in anaerobic microsites of nitrifying soils was discounted as having any influence on the $\delta^{18}$O$_{NO3}$ in these studies because a simultaneous increase in $\delta^{15}$N and $\delta^{18}$O NO$_3^-$, indicative of denitrification (Lehmann et al., 2003; Sigman et al., 2005; Granger et al., 2008) was not observed. However, some workers have found that denitrification in ground water and soils can be significant, the impact of which has been under-estimated based on interpretation of NO$_3^-$ isotopes in many systems (Houlton et al., 2006; Osaka et al., 2010), particularly as they can decouple N and O isotopes otherwise apparent for denitrification alone (Osaka 2010). Therefore, rather than the hypotheses put forth in these studies (Burns, 2002; Mayer et al., 2001; Spoelstra et al., 2007), we suspect that denitrification, co-occurring with nitrification, may cause the $\delta^{18}$O of NO$_3^-$ in some freshwater environments to fortuitously conform to, or be greater than predicted by Eq. 2.
Use of the fractional source contribution model estimate for $\delta^{18}$O$_{NO_3}$, thereby ignoring kinetic isotope fractionation during O-atom incorporation and NO$_2^-$ exchange with H$_2$O, can lead to an overestimation of nitrification as a source of NO$_3^-$ in natural systems. We therefore sought an alternative model that could be used as a more accurate, but simple, representation of nitrified NO$_3^-$ in the environment. We noticed that for all of our incubations with natural abundance H$_2$O, the $\delta^{18}$O of final NO$_3^-$ were all within a few ‰ of $\delta^{18}$O$_{H_2O}$. All treatments in Experiment 1 were lighter than $\delta^{18}$O$_{H_2O}$ by approximately 1‰, and treatments 1 and 2 for Experiment 2 were lighter by 2.2‰ and 1.4‰ respectively. In this vein, similar empirical correlates have emerged from studies of nitrifying cultures and from observations in marine systems: Buchwald et al. 2012 found the $\delta^{18}$O of newly produced NO$_3^-$ to consistently be within

![Figure 9. Density distribution of possible $\delta^{18}$O NO$_3^-$ values at a $\delta^{18}$O H$_2$O of -5‰. Calculated from Eq.8 using varying O-incorporation isotope effects and exchange values that span the entire range observed in nitrifying cultures.](image)
a few \( \delta^{18} \text{O}_\text{H}_2 \text{O} \). \( \delta^{18} \text{O} \) values for nitrified \( \text{NO}_3^- \) close to that of \( \text{H}_2 \text{O} \) are consistent with the expectation of remineralized \( \text{NO}_3^- \) in various marine systems (Casciotti et al., 2002; Granger et al., 2013; Rafter et al., 2013; Sigman et al., 2009), which have been used to interpret \( \delta^{18} \text{O}_\text{NO}_3^- \) in biogeochemical models (Casciotti et al., 2007; Sigman et al., 2005; Wankel et al., 2007). To test the hypothesis that \( \delta^{18} \text{O} \) of \( \text{NO}_3^- \) produced by freshwater nitrification is close to that of \( \text{H}_2 \text{O} \), we created a density distribution of all plausible \( \delta^{18} \text{O}_\text{NO}_3^- \) produced at \( \delta^{18} \text{O}_\text{H}_2 \text{O} = -5\%_\circ \). Calculations of hypothetical \( \delta^{18} \text{O}_\text{NO}_3^- \) consisted of all permutations of (a) O-atom incorporation isotope effects covering the range observed in cultures, and (b) varying \( X_{\text{NO}_2^-} \) at 0%, 25%, 50%, 75%, and 100% \( \text{NO}_2^- \) O-atom equilibration (Fig. 9). Of the 3,360 possible combinations of isotope effects and equilibration, over 60% were within \( \pm 3\%_\circ \) of \( \delta^{18} \text{O}_\text{H}_2 \text{O} \). Therefore, we propose that field studies of nitrification and \( \text{NO}_3^- \) source apportioning studies in freshwater environments take O-atom incorporation isotope effects and \( \text{NO}_2^- \) exchange into account by adopting the model that the \( \delta^{18} \text{O} \) of nitrified \( \text{NO}_3^- \) is approximately equal to that of the \( \delta^{18} \text{O}_\text{H}_2 \text{O} \). Although the O-atom incorporation isotope effects and amounts of \( \text{NO}_2^- \) exchange may vary widely from system to system, adoption of this convention will give a more accurate representation of the \( \delta^{18} \text{O}_\text{NO}_3^- \) produced by nitrification than that of 2:1 fractional source contribution.

4.5 Future Directions ---

Observations of \( \delta^{18} \text{O} \) of \( \text{NO}_2^- \) and \( \text{NO}_3^- \) clearly show that the O isotope dynamics of stream water nitrification align with the model proposed by the Casciotti and Buchwald culture studies (Casciotti et al., 2010; Buchwald and Casciotti, 2010; Buchwald et al., 2012). Our study further provides evidence that the rule of thumb used as the expectation for nitrification in marine
systems, that $\delta^{18}$O of nitrified NO$_3^-$ is close to that of H$_2$O, is also applicable in freshwater systems. While some important aspects of freshwater nitrification have been resolved, some queries still remain undetermined, including: How much NO$_2^-$ O atom exchange occurs in the environment, where NO$_2^-$ concentrations and residence times are low? What governs organism- catalyzed exchange? How influential is the $\delta^{18}$O of ambient O$_2$? With the answers to these questions, workers could ultimately arrive at the central question of: Can one reliably tease out the contribution of nitrification from other NO$_3^-$ sources, such as atmospheric deposition and fertilizer, and from the influence of denitrification from NO$_3^-$ N and O distributions in the environment?
References


