A chemical method for the conversion of nitrate to nitrous oxide for isotopic analysis

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1 METHOD OVERVIEW

The nitrate (NO_3^-) isotope method described here is a modified version of published chemical denitrification methods (e.g. McIlvin and Altabet, 2005; Ryabenko et al. 2009) that determine the $\delta^{15}N$ and $\delta^{18}O$ values of nitrate by sequentially converting nitrate to nitrous oxide gas (N_2O) and measuring its isotopic composition. To summarize, samples containing 2 µg of NO_3^--nitrogen (N) are freeze dried and then redissolved in a 3 mL sodium chloride – imidazole solution in a 3.7 mL Exetainer® vial. Following redissolution, activated cadmium is added to each vial to reduce nitrate to nitrite over a 24-hour period. Samples are then syringe filtered into helium-filled 20 mL serum vials and buffer solution (acetic acid and sodium azide) added to convert nitrite to nitrous oxide. After allowing the reaction to proceed to completion, sodium hydroxide solution is added to quench the reaction. Depending on the instrument used for the isotopic analysis, nitrous oxide from the headspace of the vials can be directly injected to the instrument or sub-sampled into a suitable autosampler vial. The isotopic composition of the original nitrate is determined by creating a correction equation using nitrate standards included in each run. The correction accounts for method blank effects, isotopic fractionation during the various reactions, oxygen exchange between water and NOx, and the fact that one N of the N_2O comes from the original nitrate and one from azide. Replicate samples and standards within each run are typically within ±0.5‰ or less for both $\delta^{15}N$ and $\delta^{18}O$.

2 RÉSUMÉ

La méthode d’analyse des isotopes du nitrate (NO_3^-) que nous décrivons ici est une version modifiée d’autres méthodes de dénitrification chimique ayant fait l’objet d’une publication (voir p. ex. McIlvin et Altabet [2005]; Ryabenko *et al.* [2009]); elle permet de déterminer les teneurs en $\delta^{15}N$ et en $\delta^{18}O$ du nitrate en convertissant de manière séquentielle le nitrate en oxyde de diazote (N_2O) et en mesurant la composition en isotopes du nitrate. En résumé, les échantillons contenant 2 µg d’azote nitrique (N) sont lyophilisés, après quoi ils sont dissous à nouveau dans 3 mL d’une solution de chlorure de sodium – imidazole dans un flacon Exetainer® de 3,7 mL. Après la dissolution, du cadmium activé est ajouté dans chaque flacon pour réduire le nitrate en nitrite durant une période de 24 heures. Les échantillons sont ensuite filtrés à l’aide d’une seringue dans des flacons à sérum de 20 mL remplis d’hélium, après quoi une solution tampon (acide acétique et trinitrure(1-) de sodium) est ajoutée pour convertir le nitrite en oxyde de diazote. Une fois la réaction terminée, une solution d’hydroxyde de sodium est ajoutée pour bloquer la réaction. Selon l’instrument utilisé pour l’analyse des isotopes, l’oxyde de diazote provenant de l’espace de tête des flacons peut être injecté directement dans l’instrument ou sous-échantillonné dans un flacon d’autoéchantillonneur approprié. La composition en isotopes du nitrate d’origine est déterminée par la création d’une équation de correction au moyen d’analysats de nitrates dans chaque essai. La correction tient compte des effets de la méthode des essais à blanc, du fractionnement isotopique durant les diverses réactions, de l’échange d’oxygène entre l’eau et les NO_x, et du fait que l’un des N du N_2O provient du nitrate d’origine alors que l’autre N provient du trinitrure(1-) de sodium. Les teneurs en $\delta^{15}N$ et en $\delta^{18}O$ des échantillons et des analysats répétés dans chaque essai se situent habituellement à ± 0,5 % ou moins l’une de l’autre.

3 MATERIALS AND EQUIPMENT

Exetainers®: Labco Limited, Exetainers® with caps and septa, 3.7 mL (Product code: 778W)
Serum vials: Restek headspace crimp vials, 20 mL (Product code: 21162)
Crimps: Wheaton® aluminum seals, tear-out center, 20 mm (Product code: 224183-01)
Stoppers: Wheaton® straight plug rubber stoppers, 13x20 mm (Product code: 224100-175)
Syringes: Becton Dickinson 3 mL syringe, Luer-Lok™ tip (Product code: 309585)
Filters: Millex®-LCR 0.45 µm filter, PTFE, 13 mm, non-sterile (Product code: SLCR013NK)
Needles: Becton Dickinson needle, 25 G 1½” (Product code: 305127)
Becton Dickinson needle, 23 G 1½” (Product code: 305194)
Becton Dickinson needle, 21 G 2” (Product code: 305129)
Scoop: Scienceware® Mini sampler spoon, 0.10 mL (Product code: 367210010)
Shaker: Glas-Col® rotary shaker (Product code: 099A RD4512)

4 SAMPLE COLLECTION AND PRESERVATION

The volume of sample required for nitrate (NO_3^-) isotope analysis by the chemical denitrifier method depends on the NO_3^- concentration of the sample and the requirements of the instrument used for the isotope ratio analysis. To ensure sufficient NO_3^--nitrogen (N) for most systems, 2 µg N is analyzed for each replicate of sample and standards. For samples with a nitrate concentration of >0.1 mg N/L, the collection of 120 mL of sample will be sufficient to allow for multiple runs in duplicate if necessary. Required volumes (for one analysis) can be calculated using Equation 3 (see Section 7.1 for more details). Samples should be filtered to 0.45 µm during or shortly after collection. Samples should be kept cold in the field and then stored frozen until further processing.

5 PREPARING REAGENTS AND STANDARDS

All solutions should be made with N-free deionized water (DI) and stored in tightly capped bottles. If desired, larger volumes of the solutions can be made by modifying the instructions given below.

5.1 Reagents

Table 1, Reagents required for the reduction procedures.

Compound	Formula	Molecular Weight (g/mol)
Imidazole	$C_3H_4N_2$	68.077
Sodium Chloride	NaCl	58.443
Sodium Azide	NaN_3	65.010
Sodium Hydroxide	NaOH	39.998
Helium (UHP)	He	4.003
Cadmium (325-mesh)	Cd	112.41
Acetic acid	$C_2H_4O_2$	60.05

It is the responsibility of all users to read the MSDS sheets for each chemical used in this method and be familiar with the hazards, safe-handling procedures, and personal protective equipment required.

5.2 Solutions

5.0 M NaCl, 0.02 M imidazole: Dissolve 146.1 g NaCl and 0.68 g of imidazole in 500 mL of Milli-Q® DI on a magnetic stirrer.

2 M NaN_3: Dissolve NaN_3 in DI-H_2O as indicated by Equations 1 and 2 [don't make until needed (Section 11)]:

$$\text{Volume of DI} = \#\text{ of samples} + 10\ (\text{mL}) \qquad \text{(Eqn. 1)}$$

$$\text{Mass of NaN}_3 = \text{Volume from Eqn. 1(mL)} \times 0.1300(\text{g/mL}) \qquad \text{(Eqn. 2)}$$

20% Acetic acid: RICCA Chemical Company®; Acetic Acid 20% (V/V) (Product code: 13832)

6.0 M NaOH: Dissolve 48 g NaOH in 200 mL of DI. NaOH should be added slowly while solution is mixed using a magnetic stirrer to prevent excessive heating of the solution.

To remove any dissolved nitrous oxide (N_2O) initially present, all solutions are bubbled with ultrapure helium for 15 minutes prior to using. Note, the acetic acid and azide solutions are bubbled with helium after they are combined to make the buffer solution (see Section 11 for more details).

5.3 Activation of cadmium powder

The cadmium powder, which catalyzes the reduction of nitrate to nitrite, is activated with 10% hydrochloric acid.

1) Put about 200 mL of 10% HCl in a beaker.
2) Using a scoop or lab spoon, sprinkle the cadmium powder into the HCl. Repeat this step until the desired amount of cadmium has been added.
3) After the cadmium has settled to the bottom of the beaker, stir. The cadmium will clump together.
4) Pour off the HCl into an appropriate waste container.
5) Refill the beaker with Milli-Q® DI, stir, and let sit for several minutes.

6) Pour off the DI into an appropriate waste container and refill with new DI. Let the cadmium soak in the DI for about 30 minutes and then repeat the DI rinse for a total of three 30-minute rinses.

7) Dry the cadmium overnight in an oven at about 60˚C.

8) Once dry, use a mortar and pestle to grind the cadmium into a fine powder. Store in an air-tight vial until needed.

5.4 $\delta^{15}N\text{-}NO_3^-$ and $\delta^{18}O\text{-}NO_3^-$ Standards

At least three standards are analyzed with each batch of samples. These standards include international nitrate isotope standards (e.g. USGS-34, USGS-35, IAEA-NO3) and internal nitrate isotope standards (e.g. EGC-1, EGC-16, EGC-17) that were previously calibrated against the international nitrate isotope standards.

Each of the nitrate isotope standards is prepared as a 20 mg N/L solution. At this concentration, 100 µL of standard solution is freeze dried in 3.7 mL Exetainers® for the procedure (see Section 8). Standard stock solutions are kept refrigerated.

6 CLEANING OF GLASSWARE AND EQUIPMENT

Since the amount of N_2O produced from the samples and standards is very small, it is crucial that all glassware, reagents, and equipment be free of other NO_3^-, NO_2^-, and N_2O. Glassware and non-metallic equipment used in this method are cleaned using the procedures described below. The cleaning procedure for syringes, filters, and needles is given in Section 10.

1) Rinse items with tap water to remove particulates and residual solutions.

2) Soak items in lab soap bath overnight.

3) Remove items from soap bath, scrub with brush, and rinse off soap residue with cold tap water.

4) Place items in a 10% HCl bath overnight.

5) Remove items from HCl bath and place in a bath of fresh DI water for at least 15 minutes.

8) Remove items from DI bath then rinse thoroughly with Milli-Q® DI.

9) Place items on clean lab bench paper to air dry. Alternatively, items can be oven dried. The grey stoppers must be oven dried at 60˚C for at least 24 hours to drive off any N_2O contained in the rubber.

10) Once clean, keep all equipment in designated bins with lids, separate from other glassware and supplies.

New Exetainers® and serum vials are cleaned using the procedure below. Note, the Exetainer® caps and septa do not go through the HCl bath.

1) Rinse items with DI water. Allow to soak in a Milli-Q® DI water bath overnight.
2) Remove items from DI water bath. Place in a 10% HCl bath overnight.
3) Remove items from HCl bath and place in a bath of fresh DI water for at least 15 minutes.
4) Remove items from DI bath, and rinse thoroughly with Milli-Q® DI
5) Oven-dry items at 60˚C. Note, the Exetainer® caps (with septa) must be oven dried at 60˚C for at least 24 hours to drive off any N_2O contained in them. Once clean and dry, keep all equipment in designated bins with lids, separate from other glassware and supplies.

7 PRE-FREEZE DRYING CALCULATIONS

Ideally, approximately 2 µg of NO_3^--N is processed to ensure sufficient N_2O for isotopic analysis. The volume of sample used is calculated using Equation 3 below.

Sample Volume

1) Using Equation 3, calculate volume of sample (V_s) that contains 2 µg (0.002 mg) NO_3^--N required for each vial.

$$\text{Sample Volume } (V_s) \text{ Required (in L)} = \frac{\text{Target } NO_3^- \text{ Amount } (\text{mg N})}{\text{Sample } NO_3^- \text{ Concentration } (\text{mg N/L})}$$

$$V_s = \frac{0.002\,(\text{mg N})}{[NO_3^-](\text{mg N/L})} \qquad \text{(Eqn. 3)}$$

8 FREEZE-DRYING

1) Sample volumes >1 mL should be freeze-dried in a plastic bottle of appropriate size to avoid cracking of the Exetainers® during freezing. Sample volumes <1 mL can be freeze-dried directly in the 3.7 mL Exetainers®.
2) Once dry, remove the samples from the freeze-dryer and cap tightly until ready to proceed.

9 REDUCTION OF NITRATE TO NITRITE

1) Re-dissolve samples using 3 mL of the NaCl/imidazole solution and place samples on a rotating mixer at ~12 rpm for 30 minutes (Figure 1).
2) Transfer sample to a 3.7 mL Exetainer® (Figure 2A) if they are not already in one.

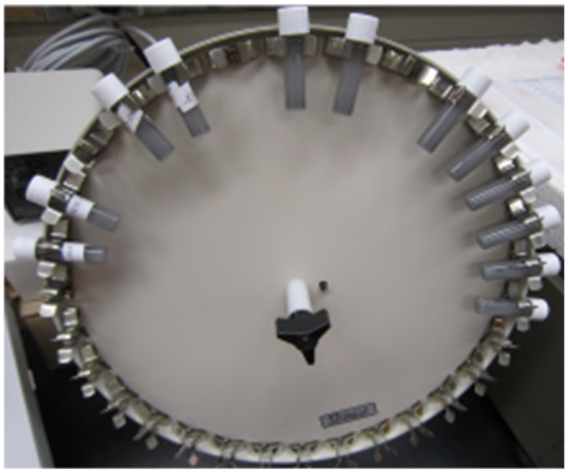

Figure 1, Exetainers® secured on rotating mixer.

3) In a fume hood, carefully add 1 scoop of activated cadmium powder to the Exetainer® and cap tightly.

4) Once cadmium has been added to all the vials, wipe down the exterior of the Exetainers® with a damp lab wipe to remove any cadmium powder.

5) Place Exetainers® securely in the rotating mixer and mix at 12 rpm for 24 hours (Figure 1).

10 REDUCTION OF NITRITE TO NITROUS OXIDE

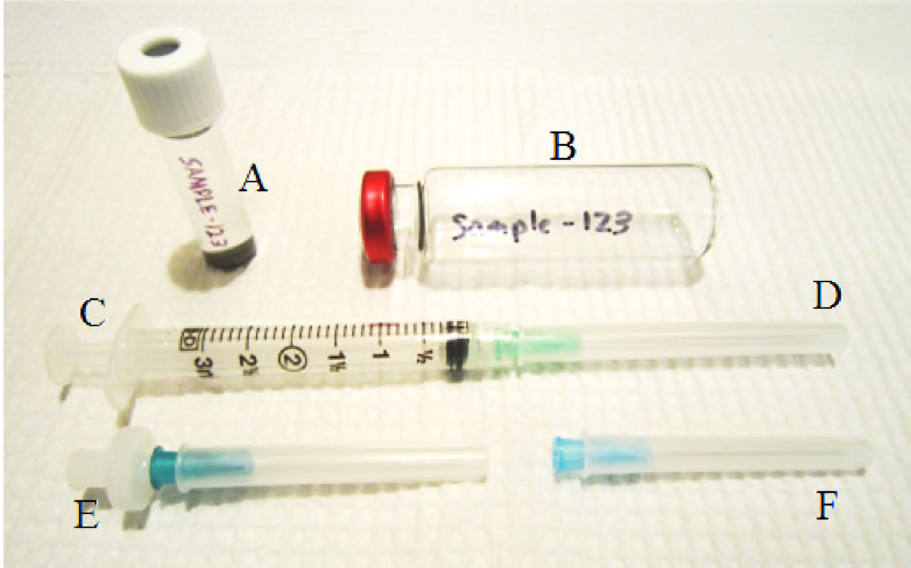

Figure 3, Equipment required to filter and transfer each sample solution after cadmium reduction step. Exetainer® with cadmium and imidazole/salt solution after 24 hours of nitrate reduction (A), 20 mL serum vial filled with helium (B), 3 mL syringe (C) with a 2" 21 G needle (D), filter-needle assembly (0.45 µm filter and 1.5” 23 G needle) (E), and 1.5” 25 G vent needle (F).

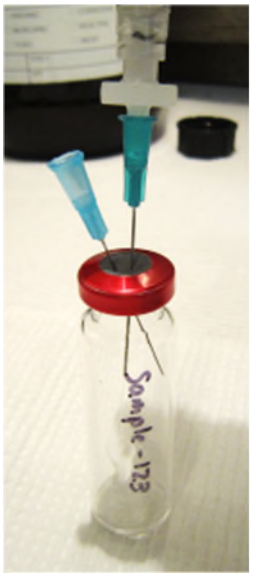

Figure 2, Injecting the solution through filter-assembly and into serum vial with vent needle in place.

1) Clean 3 mL syringes, 2” 21 G needles, and 1.5” 23 G needles attached to syringe-tip filters (Figure 2) by rinsing with Milli-Q® DI and then shaking out excess water. Allow equipment to air dry overnight.

2) Fill 20 mL serum vials with Ultra-pure helium gas to atmospheric pressure. Use aluminum crimps to secure the rubber caps onto the vials (Figure 2B)

3) Remove Exetainers® containing the samples and cadmium from the rotating mixer.

4) Using the previously cleaned 3 mL syringe (Figure 2C) and a 2” 21 G needle (Figure 2D), remove the solution from the Exetainers® and pull in a bit of air to clear the needle.

5) Cap and remove the needle and replace with the cleaned syringe-filter-needle assembly (Figure 2E). Discard the used 2” needle in an appropriate sharps container.

6) Push the 25 G vent needle (Figure 2F) and the syringe-filter-needle assembly through the septa of the helium-filled 20 mL serum vial and force the sample through the filter and into the serum vial (Figure 3).

7) Remove vent needle and syringe-filter-needle assembly from serum bottle. Cap the needles and dispose of equipment in appropriate sharps disposal containers.

11 PREPARE THE BUFFER SOLUTION

1) Using Equation 1, calculate the amount of Milli-Q® DI required for the entire run. Add this amount to an appropriately sized, pre-cleaned bottle.
2) Using Equation 2, add the appropriate amount of sodium azide to the previously measured Milli-Q® DI.
3) In a fumehood, add an equivalent volume of 20% acetic acid to the sodium azide solution and gently mix.
4) Using a gas diffuser in the fume hood, bubble the buffer solution with ultrapure helium gas for 15 minutes.

12 CONVERSION OF NITRITE TO NITROUS OXIDE

1) Transfer all or some of the buffer solution to the designated 40 mL vial with septa cap.
2) Attach a 1.5” 23 G needle to a clean 3 mL syringe, flush the syringe with helium several times, and fill with 2 mL of helium.
3) Invert the vial containing the buffer solution, inject the helium, and remove 2 mL of buffer solution. Ensure no bubbles remain in the syringe.

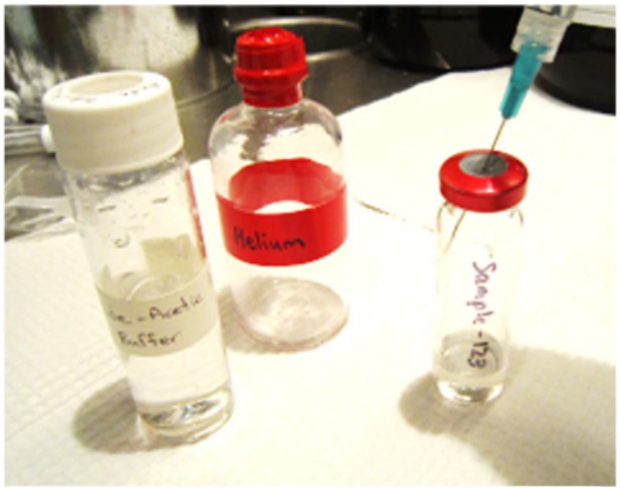

Figure 4, Injecting the acetic acid - azide buffer. Note that a vent needle is not used in this step.

4) Inject the 2 mL of buffer into the sample vial (Figure 4). Note that no vent needle is used.
5) Keeping the plunger of the syringe firmly pressed, remove the buffer syringe and needle.
6) Gently mix the sample vial.
7) Remove and inactivate any residual buffer from the syringe by flushing it into a waste beaker of DI + 1 mL 6 M NaOH.
8) Repeat steps 2 to 7 for each standard and sample vial in the run.
9) Place all serum vials on an orbital shaker for 15 minutes at 80 rpm to ensure the reaction has gone to completion.
10) Add 1 mL of 6 M NaOH to each serum vial employing the same technique used for the addition of the buffer solution.

11) Mix the serum vials thoroughly. Before subsampling for isotopic analysis, let vials sit for at least one hour to allow the dissolved N_2O to equilibrate with the headspace.
12) Wash the exterior of the serum vials with DI to remove any chemicals that might be present.
13) Inactivate any remaining buffer solution by making it basic with the addition of 6 M NaOH solution.

At this point, the vials are ready for isotopic analysis of the N_2O. Several methods can be used to introduce the N_2O to the isotope analyzer, which will depend on the instrumentation available for the $\delta^{15}N$-N_2O and $\delta^{18}O$-N_2O analysis. As is, the headspace of the vials has enough excess pressure to allow for the removal of just under 3 mL of gas, which would contain about 19 nmol of N_2O. If more N_2O is required for the isotope analyses, additional helium can be injected into the vials ahead of time. If this is done, sufficient time must be given for the dissolved N_2O to equilibrate with the headspace. Any equilibrium isotopic effects due to the partitioning of the N_2O between the liquid and headspace in the vials is corrected for in the final data correction step since the effect is the same for both the samples and standards in the run.

Currently, our method involves transferring 0.75 mL of headspace by syringe and needle to a 12 mL Exetainer® filled with 20 mL of ultrapure helium. N_2O isotope ratios are subsequently determined using a Thermo Finnigan™ GasBench™ - PreCon™ trace gas concentration system interfaced to a Thermo Scientific™ Delta V Plus™ isotope-ratio mass spectrometer.

13 CORRECTION OF THE ISOTOPE DATA

In order to calculate the $\delta^{15}N$ and $\delta^{18}O$ values of the original sample NO_3^-, a linear correction based on the standards within the run (Figure 5, 6) is applied to all samples. The correction accounts for:
- method blank effects
- fractionation effects due to N_2O partitioning between the liquid and headspace of the vials
- the fact that half of the N atoms come from the azide
- kinetic fractionation effects during the conversion of NO_3^- to N_2O
- oxygen exchange with water during the conversion of NO_3^- to N_2O

Note that no correction is needed to account for the $\delta^{18}O$-H_2O composition of the sample because all sample water is removed during the freeze drying process.

Initial method testing (not shown) indicated that the correction was linear over a large $\delta^{15}N$ and $\delta^{18}O$ range and therefore 2-3 standards for each isotope ratio are typically used (Figures 5-8 show a run with two standards each).

A second set of plots, based on Figures 2 and 3 in McIlvin and Altabet (2005) is created to help track the performance of the reduction method (Figure 7, 8). These plots use the same data as Figures 5 and 6, however the axes are switched.

The slope of the $\delta^{15}N$ plot (Figure 7) would ideally be 0.5, since half of the N atoms come from the sample NO_3^- and half from the azide reagent. However, values of the slope are typically around 0.45 due to a small method blank effect. Lower values may indicate N contamination of the reagents or equipment.

For the $\delta^{18}O$ plot (Figure 8), the slope indicates the fraction of the $\delta^{18}O$-N_2O value that is controlled by the $\delta^{18}O$ value of original sample NO_3^-. The difference (1-slope) consists of oxygen added via oxygen exchange that occurs between nitrite and water. Typical values for the slope are 0.90 to 0.95, indicating 5 to 10% of the oxygen coming from exchange with water. Additionally, the intercept of the linear equation is the net enrichment factor between N_2O and the original NO_3^- ($\varepsilon_{N_2O\text{-}NO_3^-}$) for the reduction reaction. The intercept values are typically between +35 to +38‰.

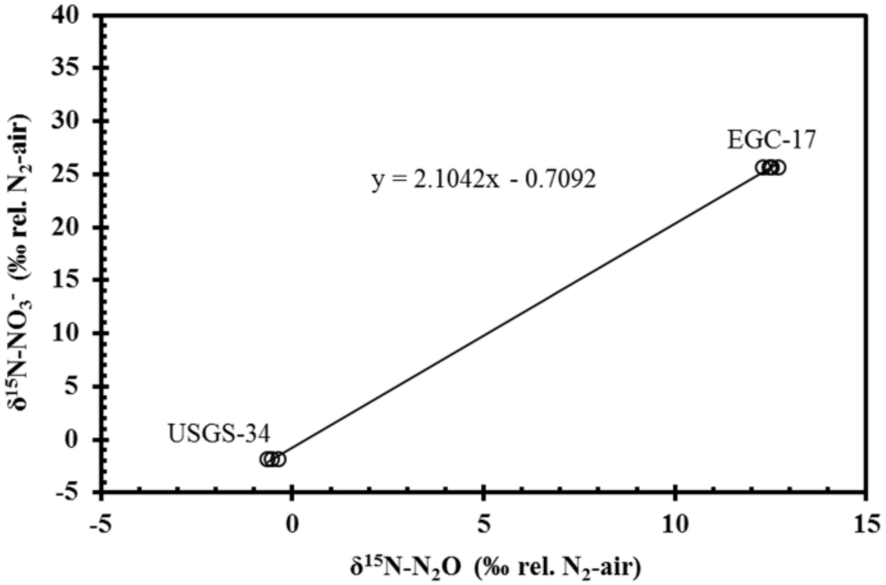

Figure 5. Linear correction plot and equation used to calculate δ^{15}N-NO_3^- values from the δ^{15}N-N_2O data of the NO_3^- isotope standards that were processed along with the samples during the chemical reduction process.

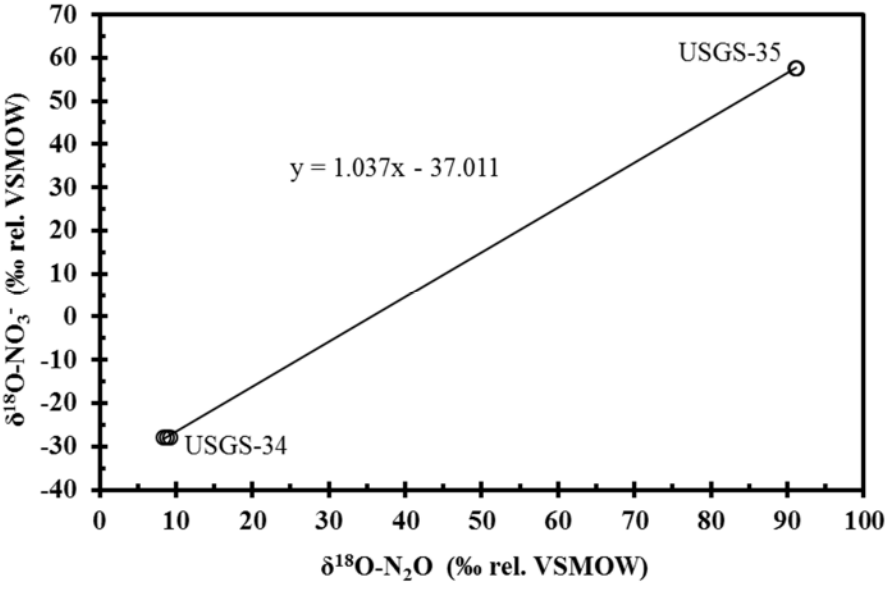

Figure 6. Linear correction plot and equation used to calculate δ^{18}O-NO_3^- values from the δ^{18}O-N_2O data of the NO_3^- isotope standards that were processed along with the samples during the chemical reduction process.

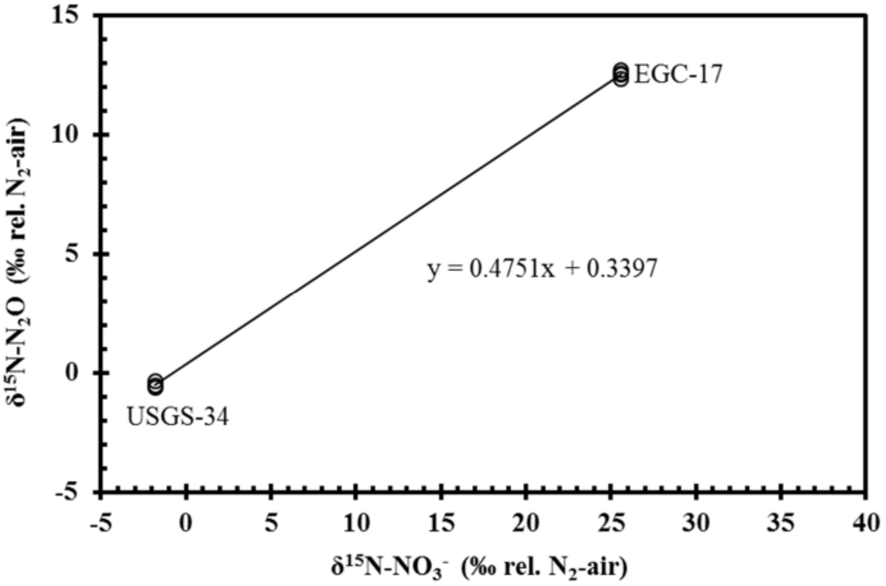

Figure 7. A linear plot of $\delta^{15}N$-N_2O versus $\delta^{15}N$-NO_3^- values (axes switched from Figure 5). Ideally, a slope of 0.5 is expected since one of the N atoms in the N_2O comes from azide and the other from NO_3^-. Values slightly less than 0.5 indicate a small blank effect. See McIlvin and Altabet (2005) for more details.

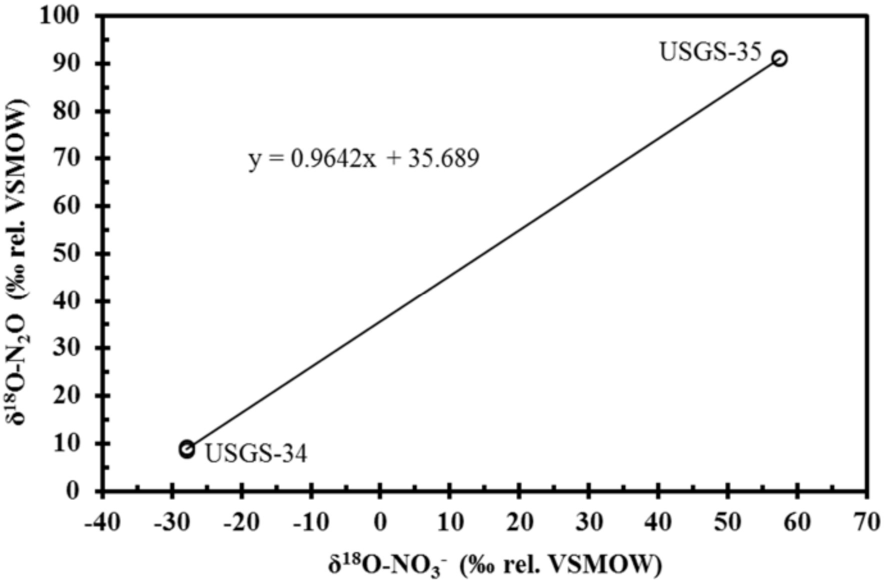

Figure 8. A linear plot of $\delta^{18}O$-N_2O versus $\delta^{18}O$-NO_3^- values (axes switched from Figure 6). The slope of the line provides information on the amount of oxygen exchange between NO_2^- and H_2O during the reduction process. The intercept value corresponds to the net enrichment factor between NO_3^- and N_2O. See McIlvin and Altabet (2005) for more details.

14 RECOMMENDED READING

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Xue, D., Botte, J., De Baets, B., Accoe, F., Nestler, A., Taylor, P., Van Cleemput, O., Berglund, M., Boeckx, P. 2009. Present limitations and future prospects of stable isotope methods for nitrate source identification in surface- and groundwater. Water Research 43, 1159–1170.

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