Received: 15 February 2019 | Revised: 1 April 2019 | Accepted: 5 April 2019
DOI: 10.1002/rcm.8454

RESEARCH ARTICLE

WILEY Rapid Communications in Mass Spectrometry

A Ti(III) reduction method for one-step conversion of seawater and freshwater nitrate into N_2O for stable isotopic analysis of $^{15}N/^{14}N$, $^{18}O/^{16}O$ and $^{17}O/^{16}O$

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Rationale: The nitrogen and oxygen ($\delta^{15}N$, $\delta^{18}O$, and $\delta^{17}O$ values) isotopic compositions of nitrate (NO_3^-) are crucial tracers of nutrient nitrogen (N) sources and dynamics in aquatic systems. Current methods such as bacterial denitrification or Cd-azide reduction require laborious multi-step conversions or toxic chemicals to reduce NO_3^- to N_2O for ^{15}N and ^{18}O isotopic analyses by isotope ratio mass spectrometry (IRMS). Furthermore, the ^{17}O composition of N_2O cannot be directly disentangled using IRMS because ^{17}O contributes to mass 45 (^{15}N).

Methods: We describe a new one-step chemical conversion method that employs Ti(III) chloride to reduce nitrate to N_2O gas in septum sample vials. Sample preparation takes only a few minutes followed by a 24-h reaction producing N_2O gas (65–75% recovery) which partitions into the headspace. The N_2O headspace was measured for ^{15}N, ^{18}O and ^{17}O by IRMS or laser spectrometry.

Results: IRMS and laser spectrometric analyses gave accurate and reproducible N and O isotopic results down to 50 ppb (3.5 μM) NO_3-N, similar in precision to the denitrifier and Cd-azide methods. The uncertainties for dissolved nitrate reference materials (USGS32, USGS34, USGS35, IAEA-NO_3) were ±0.2‰ for $\delta^{15}N$ values and ±0.3‰ for $\delta^{18}O$ values using IRMS. For laser-based N_2O isotope analyses the results were similar, with an $\delta^{17}O$ uncertainty of ±0.9‰ without any need for ^{15}N correction.

Conclusions: Advantages of the Ti(III) reduction method are simplicity, low cost, and no requirement for toxic chemicals or anaerobic bacterial cultures. Minor corrections may be required to account for sample nitrate concentration variance and potential chemical interferences. The Ti(III) method is easily implemented into laboratories currently using N_2O headspace sampling apparatus. We expect that the Ti(III) method will promulgate the use of N and O isotopes of nitrate in important studies of nutrient dynamics and pollution in a wide range of aquatic ecosystems.

1 | INTRODUCTION

Excessive concentrations of nitrate (NO_3^-) stemming from agriculture runoff and municipal wastewater entering rivers, lakes, groundwater, and marine estuaries have resulted in increasingly widespread negative impacts that include eutrophication, dead zones in coastal oceans, and drinking water deterioration in many worldwide aquifers.[1-4] Hence, identifying and quantifying the sources and biogeochemical processing of nitrate in aquatic systems are key to informing water resource management, agricultural nutrient optimization strategies, and urban pollutant mitigation.[5-7] Stable isotopes are proven tools in this regard, as the $\delta^{15}N$, $\delta^{18}O$ and $\delta^{17}O$ isotopic composition of NO_3^- provides essential information about nitrogen (N) sources and biogeochemical sinks, allowing one to distinguish between organic waste, fertilizers, and atmospheric N sources.[8-14] Furthermore, isotopic time series and geospatial depictions of nitrate in ground and surface water reveal important changes in agricultural land use, inform optimization of fertilization practices not achievable based on NO_3^- concentrations alone, and allow for detection of natural bioremediation processes such as bacterial denitrification.[15,16] NO_3^- stable isotopes have also been instrumental in studies of the natural N cycle in a variety of systems including the global ocean and large-scale variations in NO_3^- $\delta^{15}N$ and $\delta^{18}O$ values have been used to understand the ocean N balance between losses from denitrification and gains from N_2 fixation.[10,17-19] Unfortunately, for decades, nitrate isotopic assays have been laborious and costly because of the difficulty of quantitatively extracting dissolved NO_3^- from water with subsequent conversion into N_2, CO or N_2O gas for N or O isotope-ratio mass spectrometry (IRMS), thereby hampering wide-spread adoption and limiting scientific accessibility.[20] Previous offline NO_3^- extraction and conversion methods included Kjeldahl distillation for $\delta^{15}N$ values,[21] and ion-exchange recovery or micro-diffusion and precipitation to a nitrate salt[22,23] for $\delta^{15}N$ and $\delta^{18}O$ determinations by pyrolysis-IRMS analysis. Contemporary methods for IRMS or laser analysis largely favor conversions of NO_3^- into N_2O gas using the bacterial denitrifier[24-27] or the Cd-azide reaction methods.[28-30] Both methods, however, either require laborious multi-step conversion methods and careful maintenance of anaerobic bacterial cultures, or use highly toxic chemicals to reduce NO_3^- to N_2O for N and O isotopic analyses by IRMS and, as a result, are costly and limited to specialized isotope laboratories. In both cases, the $\delta^{17}O$ values of NO_3^- cannot be obtained directly by IRMS due to isobaric interferences between ^{15}N and ^{17}O in N_2O, unless there is subsequent conversion of the produced N_2O gas into N_2 and O_2 and a dual-inlet IRMS analysis of O_2 is undertaken.[31] New advances in isotope analysis methodology for nitrates should (i) have N_2O as an end product to facilitate dual O/N isotope analysis and cryo-focusing for high sensitivity, (ii) produce quantitative conversion of NO_3^- into N_2O gas or non-quantitative conversion with constant isotope fractionation, (iii) have a correctable or no blank, (iv) be insensitive to major ion composition ranging from fresh to marine waters, and (v) allow for assays on low NO_3^- concentrations (i.e. nmol/ppb levels in oligotrophic aquatic or ground water systems). Improved methods should also be low-cost and amenable to automation on existing instrumentation, avoiding use of toxic chemicals or maintaining bacterial cultures to help reduce technological barriers to adoption. Finally, new methods should produce accurate and appropriately precise results using IRMS or laser instruments.

NO_3^- reduction generally proceeds as $NO_3^- \Rightarrow NO_2^- \Rightarrow NO$ with N_2O, NH_4^+ or N_2 as end products with other possible short-lived intermediates.[32] Ti(III) chloride was previously used to convert NO_3^- into NO (sparged out of solution before further reduction) or NH_4^+ for colorimetric or chemiluminescent analysis,[33-37] but was never applied to stable isotope N and O assays. The reaction pathway at low pH, though, leads to N_2O as an end product,[38] ideally given by:

$$2NO_3^-{}_{(aq)} + 8Ti^{+3} + 10H^+ \rightarrow N_2O_{(g)} + 8Ti^{+4} + 5H_2O \qquad (1)$$

whereby aqueous nitrate is reduced to N_2O gas at the expense of trivalent titanium which is accordingly oxidized to tetravalent titanium.[39] A consideration in making use of this reaction for NO_3^- stable isotope analysis is that the presence of dissolved oxygen and oxy-anions (e.g. $SO_4^=$) may cause interferences, incomplete reactions or the formation of Ti dioxide precipitates.[40]

Here we propose a simple conversion method that utilizes Ti(III) chloride in a one-step conversion of aqueous nitrate into N_2O headspace gas for $\delta^{15}N$ and $\delta^{18}O$ or $\delta^{17}O$ analysis using IRMS or N_2O laser spectrometers. We report first N and O isotopic results from the Ti(III) technique and propose standardized operational procedures for NO_3^- in fresh and marine waters (see supporting information). We discuss some of the factors encountered that affected optimal N_2O recovery and identified solutions. Finally, we compare our Ti(III)-based N and O isotopic results from contaminated ground water samples with analyses of the same samples obtained using the bacterial denitrifier and Cd method. With further testing and development, we expect that the ease and low cost of the Ti(III) method will foster new N and O isotope studies of nitrate dynamics in a wider range of aquatic ecosystems.

2 | EXPERIMENTAL

2.1 | Overview

The Ti(III) method comprises (1) a sample processing and (2) an analytical component for nitrate N and O isotope determinations. The first component is a simple one-step chemical conversion of aqueous NO_3^- into headspace N_2O using a commercially available Ti(III) chloride reagent (Equation 1). The second component consists of N and O isotopic analysis of the evolved headspace N_2O gas by isotope ratio mass spectrometry or by laser spectrometry. For the sample processing component, we utilized Ti(III) chloride in a 1:20 or 1:40 (v/v) proportion to nitrate samples to convert aqueous NO_3^- into N_2O gas. Because we have found that water sample chemistry can affect N_2O yield and isotope results, we matched samples and calibration standards with respect to both ionic matrix (i.e. seawater vs freshwater) and NO_3^- concentration. For the analytical component, we used IRMS systems that employed headspace sampling followed by cryogenic and gas chromatographic purification of the N_2O from N_2, O_2, CO_2, H_2O with automated sample handling devices (i.e. Trace Gas interface). We also used a N_2O isotopic laser instrument, newly available technology that does not require cryogenic purification of N_2O for isotopic analyses and permits direct measurements of mixed gas headspace samples. A key advantage of IRMS is automation of the vial headspace N_2O

sampling for isotope analyses, but a distinctive disadvantage is the inability to measure ^{17}O in N_2O. While mass-dependent fractionation of 0.52 relative to ^{18}O is assumed for ^{17}O corrections of ^{15}N in N_2O for most environmental samples, it does not hold where NO_3^- has experienced mass-independent fractionation, typically when nitrate has atmospheric sources.[41,42] Hence, an advantage of using a N_2O triple-isotopic laser is the ability to directly measure nitrate ^{17}O via N_2O[30]; however, available laser instrumentation currently lacks automated N_2O headspace sampling devices.

2.2 | Preparation of the Ti(III) chloride reducing reagent and other materials used

The chemical reduction of aqueous nitrate to N_2O utilized Ti(III) chloride in 30% hydrochloric acid (HCl; P/N 8.08308.0500; Merck, Burlington, VT, USA). We found that the magenta colored Ti(III) chloride used "as-is" from the chemical supplier often gave variable N_2O yields, depending on the lot number or the supplier (i.e. Merck; Acros Organics; Thermo Fisher). Thus, sub-aliquots of the Ti(III) chloride reagent in enough quantity to process nitrate samples were pre-conditioned using Zn metal powder (ZN006040/7; 150 microns, 99.9% purity; Goodfellow, Huntingdon, UK) to remove potential Ti(IV) impurities from the reagent and ensure consistent reaction results. This was done by reacting 1 g of Zn powder with 10 mL of the Ti(III) chloride reagent:

$$2Zn_{(s)} + Ti^{+4}Cl_4 + 2H^+ \rightarrow 2ZnCl_{2(s)} + Ti^{+3} + H_2 \qquad (2)$$

The reaction of the acidic Ti(III) reagent with Zn was exothermic, foaming and producing H_2 gas (hence, the pre-treatment was done in a 250-mL beaker in a fume hood). After cooling to room temperature and double checking by occasional stirring to ensure that the reaction was complete, the Ti(III) chloride solution was used immediately for sample nitrate conversions into N_2O. Drawing Zn metal and precipitate from the bottom of the beaker was avoided by taking the supernatant with a micro-pipette. After use, the original Ti(III) chloride reagent bottle was flushed with N_2 or Ar gas and tightly re-sealed to avoid Ti(III) oxidation or absorption of water. The Ti(III) pre-conditioning procedure was used in all our sample preparative methods described below. When left exposed to air, the pre-conditioned Ti(III) reagent also quickly oxidizes, forming a white titanium dioxide precipitate within hours. Unused pre-conditioned reagent after sample preparation was discarded to chemical waste.

Other reagents used were 10% HCl (v/v) made using degassed deionized or distilled water. In our research we also found that some reverse-osmosis (RO)-based water systems produce water with considerable dissolved N_2O, which would contribute to high and unpredictable blanks when used as a diluent for sample preparation. We therefore recommend that the deionized water (DIW) used in preparations be tested for N_2O content or is degassed by boiling and subsequently stored for use in sealed bottles.

2.3 | Field nitrate samples and reference sample preparation

At the International Atomic Energy Agency (IAEA, Vienna, Austria), surface and ground water samples were collected by filtration through GF/F filters (0.2 or 0.45 μm) into HDPE bottles, and preserved by freezing or acidification to pH 2–3 by adding 1 mL 2.5 mM sulfanilic acid in 10% (degassed) HCl to remove NO_2^- to each 100 mL of sample. The samples were thereafter stored at 5°C or frozen (−20°C). At the University of Massachusetts at Dartmouth (UMD, New Bedford, MA, USA), filtered marine samples were stored and preserved similarly, but 0.8 mL 2.5 mM sulfamic acid in 25% HCl per 100 mL sample was used to remove NO_2^-[43] if NO_2^- was known to be present, and the acidified samples were stored at room temperature. If NO_2^- was not removed, inaccurate NO_3^- isotope results would be obtained as it is an intermediate in the reduction of NO_3^- to N_2O and thereby would also be converted into N_2O by Ti(III).

At UMD, nitrate isotope research was focused on marine and estuarine systems, typically having seawater salinity (>20–30 g/L as total dissolved solids (TDS)) and low NO_3^- concentrations. To ensure samples and reference matrix equivalence in the reaction procedures, all nitrate isotope reference solutions were made to 0.15mg L^{-1} NO_3^--N (10 μM) by dissolving or diluting with low nutrient (NO_3^--free) seawater (LNSW). Samples with known NO_3^- concentration were diluted to the target amount using LNSW. Nitrate reference materials from the United States Geological Survey (USGS, Reston, VA, USA) and the IAEA included USGS34, USGS35 and IAEA-NO_3 dissolved in LNSW with assigned $\delta^{15}N_{AIR}$ values (±SD) of −1.8 (±0.1), +2.7 (±0.1) and +4.7 (±0.1), $\delta^{18}O_{VSMOW}$ values of −27.9(±0.4), +57.5(±0.3) and +25.6 (±0.3), and $\delta^{17}O_{VSMOW}$ values of −14.6, +51.5 and +13.2, respectively.[44,45] An in-house UMD nitrate reference material for $\delta^{15}N$ values called 'Labmix' ($\delta^{15}N$ = +38.9‰) was produced by gravimetrically mixing solutions of IAEA-NO_3 with USGS32 ($\delta^{15}N_{AIR}$ = +180) in LNSW. In all instances, the use of LNSW ensured that marine or estuarine samples and the isotopic reference materials were similar in their salt type and content, including the HCl and sulfamic acid concentrations.

At the IAEA, the emphasis was on surface, groundwater and precipitation freshwater samples having comparatively higher NO_3^- concentrations than seawater but low in TDS (<3000 mg L^{-1}). The IAEA freshwater samples and references for IRMS and laser analysis were targeted to 0.2 mg L^{-1} NO_3-N (14.3 μM). Reference solutions of USGS32, USGS34, USGS35 and IAEA-NO_3 were prepared by dissolving them in DIW to 1 mg L^{-1} NO_3-N (71.4 μM), preserving them as above, and storage at 5°C in 500-mL glass bottles. The nitrate concentrations of field samples were quantified using a Discrete Analyzer (Seal AQ1; www.seal-analytical.com; US EPA Method 126-A). Before processing samples to N_2O with the Ti(III) reagent, the references and samples were gravimetrically diluted by using degassed DIW to the target concentration of 0.2 mg L^{-1} NO_3^--N (14.3 μM) in the sample reaction vial, based on NO_3^- concentration data (supporting information). Field samples with high concentrations of NO_3^- (e.g. >5 mg L^{-1} N) were pre-diluted to 5 mg L^{-1} using degassed DIW before final dilution to 0.2 mg L^{-1} and processed as above. This dilution step helped to eliminate the potential effect of interfering $SO_4^=$ (see below).

2.4 | Conversion of NO_3^- by $TiCl_3$ into N_2O

For the IRMS analyses, the target sample or reference material was pipetted into a reaction vial as described above and diluted up to 2–4 mL with LNSW or degassed DIW. At UMD, 20-mL crimped sealed headspace glass vials were used with a target of 20 to 40 nmol NO_3^-. At the IAEA, 40-mL butyl-gray septum-capped glass Exetainer vials were used, targeting 23 nmol NO_3^-. Both vials are autosampler compatible. The pre-conditioned Ti(III) chloride reagent was pipetted at a 1:20 or 1:40 (v/v) reagent-to-sample ratio and, because the Ti(III) reaction begins immediately, each sample reaction vial was immediately capped with a butyl rubber stopper or a butyl-gray septum cap followed by gentle swirling to fully mix reagent and sample. The vials containing magenta colored samples plus reagent were placed in a tray and the reduction reaction passively completed overnight for a minimum of 12–24 h (see section 3). Larger sample volumes could be accommodated to obtain the desired IRMS N_2O signal sizes for routine analyses on samples as low as 50 ppb NO_3^--N (3.5 μM).

Since each reaction vial headspace also contained atmospheric air, there was a small atmospheric N_2O blank in all samples (~0.2 nmol N_2O per 10 mL of air headspace) which was corrected later by conducting a blank subtraction (supporting information). Optionally, sample vials could be evacuated after pipetting the sample, capping and injecting Ti(III) to remove the atmospheric N_2O blank but, given the higher viscosity of the pre-conditioned Ti(III), it is more difficult to reliably inject and often splattered onto the sides and stuck to the septum cap. Hence, it was easier and more reliable to quickly dispense and cap, and conduct a blank subtraction. Because O_2 is present in the headspace of the reaction vials, progressive oxidation of magenta-colored Ti(III) to colorless Ti(IV) proceeded to form a “titanium white” precipitate.

At UMD, a typical IRMS sample preparation and analytical template consisted of two LNSW blanks (zero-nitrate) and three replicates of 10 μM USGS34, USGS35, IAEA-NO_3 and Labmix NO_3^- reference solutions at the beginning, middle and end of the run, plus up to 70 unknown NO_3^- samples. Pure NO_2^- in LNSW or DIW was reacted with sodium azide to produce N_2O[28] to quantify the comparative yield of N_2O via the Ti(III) reaction. At the IAEA, the IRMS analysis template was similar with only small differences, having up to 70 samples (described in the supporting information).

For laser spectroscopy at IAEA, batch conversion of NO_3^- into N_2O for N and O stable isotopic analyses followed a similar preparative procedure. A typical laser sample template consisted of two blanks, and triplicates of 1 mg L^{-1} N USGS and IAEA nitrate reference solutions, plus 20–30 unknown NO_3^- samples (all adjusted using DIW to a target of 0.3 mg L^{-1} NO_3^--N). The sample reaction vial for laser analyses were 125-mL Wheaton glass serum bottles (Z114014; Sigma Aldrich, St Louis, MO, USA). In each bottle, 18 mL of sample (0.3 mg L^{-1} N) was dispensed using a pipette. We then pipetted 42 mL of degassed DIW to dilute the sample and added 0.6 mL of 10% HCl to ensure that the samples were pH <2. The sample bottles were sealed using 14 mm O.D. butyl blue septa (Bellco Glassware, Vineland, NJ, USA; 2048–11800) and crimp sealed. The sample bottles were evacuated and degassed using a rotary vacuum pump connected to a 21G needle inserted though the septa (<30 mbar, approx. 2 min). After degassing and evacuation, 3 mL of the preconditioned Ti(III) chloride solution was carefully injected into the sample bottle using a gastight syringe and a 21G needle. The nitrate sample and Ti reagent were gently mixed, and each bottle was over-pressurized with 140 mL of N_2O-free air. The samples were then allowed to react for a minimum of 12–24 h to complete the reduction of NO_3^- to N_2O. Approximately 30 samples and references could be manually analyzed by laser spectroscopy per day.

2.5 | IRMS $\delta^{15}N$ and $\delta\ ^{18}O$ analyses

At UMD, an Isoprime™ continuous-flow (CF) isotope ratio mass spectrometer (Isoprime Ltd, Cheadle Hulme, UK) was used for $\delta^{15}N$ and $\delta^{18}O$ analyses of N_2O. The CF-IRMS system utilized a custom purge-and-trap (P&T) system,[28] with N_2O headspace samples extracted from 20-mL vials using a PAL autosampler (CTC, Zwingen, Switzerland). A custom double-needle was used to purge the headspace gas with N_2O into cold traps for purification. Cryogenically focused N_2O peaks were compared with those from N_2O uncalibrated reference gas pulses. The UMD sample gas handling system was fitted with a gas injection loop to allow 20 μL (STP) of 1% N_2O in N_2 to be injected into the same cryo-focus path as the samples in order to check instrumental drift, recovery and internal reproducibility.

At the IAEA, an Isoprime-100™ CF isotope ratio mass spectrometer and Trace-Gas (TG) system was used for automated $\delta^{15}N$ and $\delta^{18}O$ analyses either (i) by reacting nitrate samples in 40-mL Exetainers or (ii) by injecting N_2O headspace obtained from 125-mL laser bottles (below) into pre-evacuated 40-mL Exetainers™ (Labco Ltd, Lampeter, UK). The Isoprime TG system utilized a model GX-271 autosampler (Gilson Inc., Dunstable, UK) with a liquid N_2 P&T capillary GC system to purify and cryo-focus N_2O from 40-mL mixed gas vials. The sample N_2O peak was compared with those from uncalibrated N_2O reference gas pulses introduced by a dual-inlet bellows. We found that the delicately machined steel needle-in-needle of the Isoprime TG system quickly corroded when purging the acidic Ti(III) reagent with sample. To overcome this, the purging needle was re-positioned just above the liquid and the headspace N_2O trapping time was increased to 300 s. This eliminated corrosion of the needle while trapping the N_2O from the sample. Both Isoprime systems had an external analytical precision of better than ±0.1‰ ($\delta^{15}N$-N_2O) and ±0.2‰ ($\delta^{18}O$-N_2O).[46] An ^{17}O interference correction for the $\delta^{15}N$ value of N_2O ($\lambda = 0.52$) was applied to all samples,[28] except for USGS35 NO_3^-, a reference material with an anomalous ^{17}O content relative to ^{18}O.[41]

2.6 | Laser N_2O isotope spectrometry

At the IAEA, laser-based N_2O stable isotopic analysis ($\delta^{15}N^{\alpha}$, $\delta^{18}O$ and $\delta^{17}O$ values) was conducted using a triple isotope N_2O analyzer (23e-EP model 914–0060; Los Gatos Research, Mountain View, CA, USA) fitted with an injection port and using ‘injection-dilution’ mode. The analytical precision for 10-ppm N_2O in dry air was better than ±0.2‰ ($\delta^{15}N$-N_2O), ±0.4‰ ($\delta^{18}O$-N_2O) and ±1.0‰ ($\delta^{17}O$-N_2O). Sample analysis was performed as described in Wassenaar et al.[30] Briefly, after the 12–24 h nitrate conversion reaction into N_2O, the 125-mL serum bottles were gently swirled to ensure that the N_2O gas was fully equilibrated in the headspace. A 20-mL gastight syringe with two-way valve and needle was used to draw sample headspace gas into the barrel. As the gas overpressure of the sample pushed the plunger out, it was manually held at 3–4 mL (depending on vial initial pressure), to achieve a target 12 mL mixed headspace gas in the syringe (at STP) when the valve was closed. The syringe was taken out of the bottle, the needle removed, and fitted on the injection port of the laser instrument, and N_2O isotopic analysis was conducted. The sample analysis time for N_2O triple isotopic analysis was approximately 12 min. Normalized triple isotope δ values were processed using the algorithms described in Wassenaar et al.[30]

3 | RESULTS

3.1 | Standard operating procedures

The acceptance of new isotopic preparation and analysis methods is ultimately judged on their ease of incorporation, as well as their precision and accuracy performance. Other factors ideally include improved sample throughput, lowered costs and insensitivity to water sample matrix effects. Our Ti(III) method has few procedural steps and is low cost, which makes adoption attractive but, to optimize the isotopic results, we examined several complicating factors influencing isotope results identified during our research, including reagent-to-sample volumes, sample reaction times and sulfate interferences.

Overall, we found that the best isotopic results were obtained when pre-treated $TiCl_3$ reagent was added to nitrate samples in a 1:20 to 1:40 ratio (v/v) and reacted for 12–24 h without any agitation at room temperature (Figures 1 and 2). Below we document in detail experimental results supporting our procedures for seawater and freshwater as summarized in the supporting information. We assessed the influence of NO_3^- concentration variance and sulfate ion composition. Because of the influence on yield and isotopic results, we recommend that NO_3^- concentrations are accurately pre-determined and that sample chemistry properties be manipulated to be as similar as possible to those of the

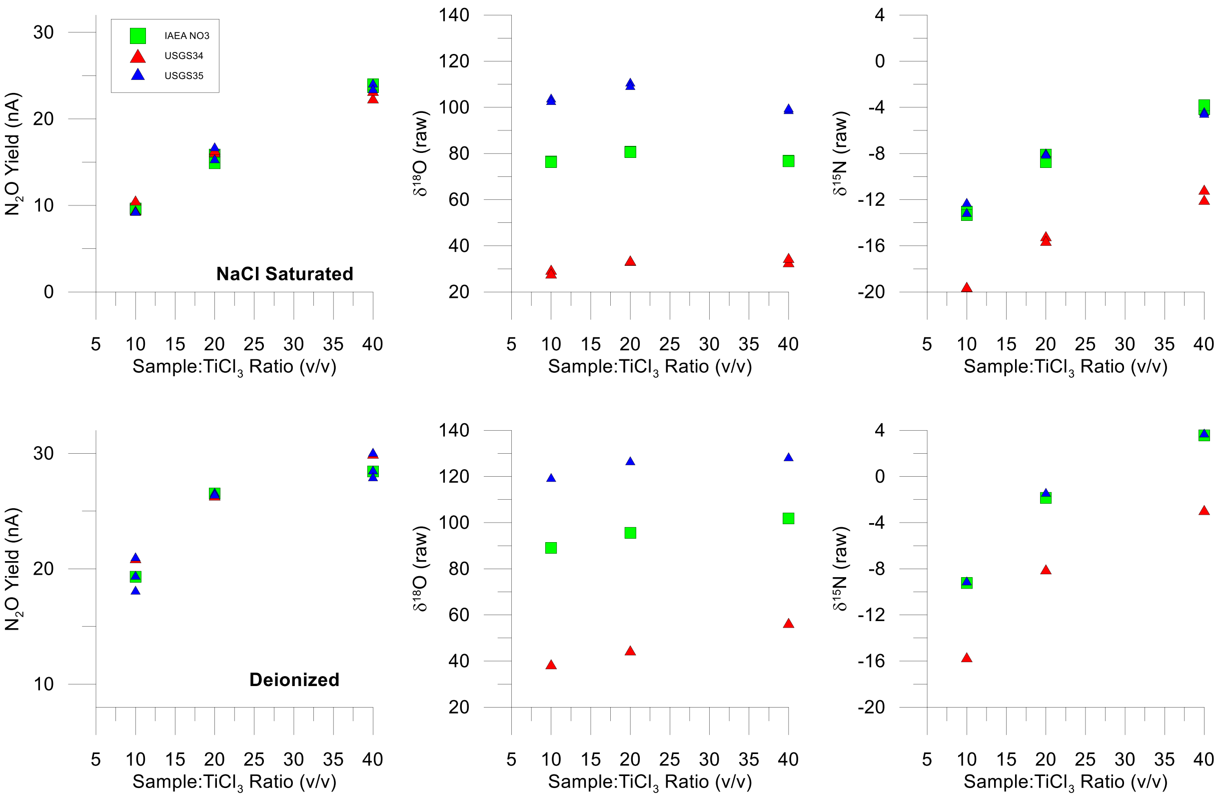

FIGURE 1 Relative N_2O signal yield (peak height nA on IRMS) and uncorrected $\delta^{15}N$ and $\delta^{18}O$ values for nitrate reference materials as a function of sample to Ti(III) reagent ratios (x-axis), ranging from 10:1 to 40:1. Upper panel is for a 6 M NaCl sample matrix and the lower panel is using DIW. For each symbol *n* = 2. Reaction time for all samples was >12 h (overnight) [Color figure can be viewed at wileyonlinelibrary.com]

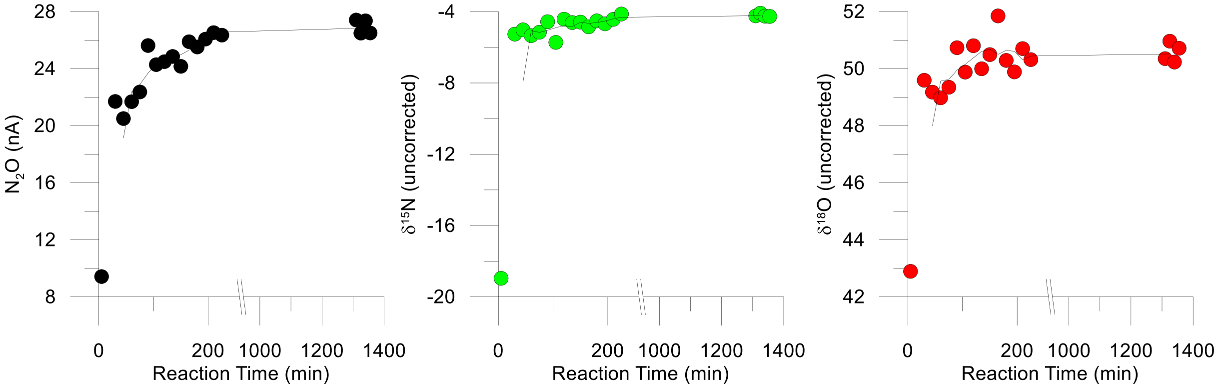

FIGURE 2 N_2O yield (nA signal on IRMS) and the uncorrected $\delta^{15}N$ and $\delta^{18}O$ compositions as a function of reaction time from 0 to 1300 min (0–21 h) using the second approach (see text) at a reagent-to-sample volume of 1:40. Stable N_2O yield and isotopic data were obtained between 4 and 21 h. Other tests (not shown) suggested that reaction times of >12 h were sufficient (e.g. overnight). Line is moving average. Note x-axis break [Color figure can be viewed at wileyonlinelibrary.com]

corresponding isotope calibration standards (e.g. saline vs freshwater).

3.2 | N_2O yields

The Ti(III) reaction produced N_2O recoveries between 65 and 80% for a reagent-to-sample volume ratio of 1:40 with DIW having the highest yield and LNSW and acidified LNSW having lower yields (Table 1). Azide reaction of NO_2^- reference solutions was used as a yield benchmark (Table 1). The recovery was found to be dependent on the Ti(III) reagent-to-sample volume ratio with higher amounts of Ti(III) reagent (i.e. 1:10 ratio) reducing N_2O recovery both in DIW and in a NaCl-saturated solution (Figure 1). The isotope results are discussed in greater detail below, but we also found that lower amounts of Ti(III) reagent (1:40 ratio) within the tested range produced slightly more precise $\delta^{15}N$ values but less precise $\delta^{18}O$ values. Conversely, the $\delta^{18}O$ values were more precise at higher Ti(III) reagent-to-sample ratios (1:10) but the $\delta^{15}N$ values less so. A ratio of between 1:20 and 1:40 was therefore deemed practical for overall general performance (see below). Despite the N_2O yield not being quantitative, the N and O isotope results were excellent within this range of reagent addition. We did not test the performance of ratios lower than 1:40 as the very small Ti(III) reagent volumes required became difficult to precisely aliquot (e.g. <75 µL) using standard micro-pipettes.

TABLE 1 N_2O recovery from NO_3^- using the Ti(III) method for reference solutions made up in different matrices. Reagent-to-sample volume ratio used was 1:40. Recovery was quantified from N_2O peak heights calibrated against azide reacted NO_2^- solutions. Corresponding isotope data is plotted in Figure 3 [Color table can be viewed at wileyonlinelibrary.com]

Matrix	NO_3^- conc. (µM)	Volume (mL)	% recovery	n	S.D.
DIW	10	2	81	8	8%
LNSW	10	2	73	16	5%
Acidified LNSW	10	2	66	8	5%

We infer that incomplete N_2O recoveries were partly due to the formation of other stable (or steady-state) end products such as N_2 or NH_4^+,[39] mainly because (i) higher Ti(III) reagent additions reduced N_2O yields and (ii) increased reaction times beyond 24 h did not show any increase in N_2O yield or better stable isotopic results. In addition, a comparative Ti(III)-azide reagent approach (not suitable for $\delta^{18}O$ values; not shown) gave ~100% N_2O yield, suggesting that the first reduction step of NO_3^- to NO_2^- by Ti(III) is quantitative. In the Ti(III)-azide reaction, NO_3^- is reduced to NO_2^- which then reacts with the azide to form N_2O before further reduction by Ti(III) can occur. Therefore, we suspect that reduced N_2O yield may be due to alternative reaction pathways leading to NH_4^+ or N_2 after the formation of NO (its production by Ti(III) reduction of NO_3^- has also been reported to be quantitative[36]) or perhaps through formation of unknown solid complexes of *N*-oxide intermediates with Ti^{+3} or Ti^{+4}. Future research considering in more detail the chemistry of Ti(III) reduction of NO_3^- may help to identify conditions resulting in better quantitative recovery of N_2O. We acknowledge that this aspect of our work requires more research.

3.3 | Reaction time, temperature, and salinity

To empirically determine the optimal reaction time for the reduction of NO_3^- to N_2O, a time-series experiment was carried out at room temperature. In one test, the reaction of a nitrate standard multiple vials with Ti(III) was terminated at different times by the addition of NaOH, and the samples were analyzed as a single batch on the next day (not shown). NaOH terminated the Ti(III) reaction because, at high pH, Ti(III) is quickly oxidized to Ti(IV), preventing nitrate reduction. In a second test, samples were sequentially analyzed by IRMS starting immediately following the addition of the Ti(III) reagent and analyzed at 12-min time-steps over a 0 to 96 h period

(Figure 2). Both approaches, however, showed that the nitrate into N_2O conversion reaction appeared complete (or stable) between 6 and 21 h with respect to N_2O yield (Figure 2). However, the N and O isotope results were found to be more precise when samples were left to react over a longer time (i.e. >12–24 h), possibly because of a longer period required for reaching gas–liquid equilibrium under static conditions. A 96-h reaction gave similar N_2O yields and isotopic results to a 21-h reaction, which suggested that samples can be reacted and held longer without adverse effects for at least 3 days before isotopic analysis. For this reason, an overnight reaction (or >12 h) was found to be convenient, so that samples could be prepared on Day 1 and analyzed for isotopes on the next morning. The long reaction periods (e.g. >96 h) visibly resulted in more Ti(IV) oxide precipitating in the bottom of the reaction vials.

The influence of reaction temperature on the Ti conversion of nitrate into N_2O was investigated by reacting samples for 12 h at 50°C and comparing the results with the room temperature results (20–22°C). No difference in the N_2O yield or isotope results was observed; hence, we recommend carrying out the reaction at room temperature to avoid unnecessary thermal control.

Tests using nitrate standards prepared in 6 M NaCl showed no adverse performance effects compared with those samples prepared using DIW (Figure 1), but some of the differences observed may account for the N_2O yield reduction in LNSW and HCl-acidified LNSW (Table 1). Comparing the salt and DIW standard preparations also showed both N_2O yield and N and O isotopic differences, but each was internally consistent and reproducible to a high degree of accuracy. This finding supported the idea that sample and reference chemistry matrices should ideally be matched. While the total dissolved salts (~35 g L^{-1} TDS) in open oceans vary narrowly in major ion composition (~3500 mg L^{-1} SO_4), 'fresh' surface and ground waters vary tremendously at lower TDS ranges, from dilute meltwater to >5000 mg L^{-1}. Ground and surface water samples also vary widely in their Cl^-, SO_4^- and HCO_3^- concentrations depending on watershed geological characteristics. We believe that $SO_4^=$ is of particular importance due to its high concentration in seawater and wide concentration variation in terrestrial freshwaters, as demonstrated previously.[40] Our tests using nitrate reference materials showed that increasing the sample sulfate concentrations from <0 mg L^{-1} to >2700 mg L^{-1} depressed the N_2O yields by about 10% at the highest concentrations (Table 2) with significant isotope effects. The $\delta^{15}N$ values were reduced by ~2‰ and the $\delta^{18}O$ values became more positive by ~2‰ over this SO_4^- range. Visually, samples with more than ~1000 mg L^{-1} sulfate also produced a secondary dark gray precipitate compared with samples with lower SO_4^- or DIW which remained magenta with a white Ti dioxide precipitate forming. The presence of HCO_3^- did not have any discernable effect on the N_2O yield or the isotopic composition.

Efforts to remove sulfate from water samples beforehand were unsuccessful. We attempted precipitation of SO_4 to $BaSO_4$ by adding $BaCl_2{*}H_2O$, and by sulfate anion-exchange cartridges (On-Guard II; Dionex, Sunnyvale, CA, USA). In both cases, sample N_2O yields and isotopic results were considerably worse, probably through co-precipitation of NO_3^- with $BaSO_4$. We did not have access to X-ray diffraction or fluorescence instrumentation to determine the composition of the visible gray co-precipitates. Our data also showed there was little effect on $\delta^{18}O$ values for samples with SO_4^- concentrations below ~600 mg L^{-1} or for $\delta^{15}N$ values at concentrations below ~300 mg L^-. The IRMS standard operating procedure (see supporting information) therefore includes a practical step for freshwater samples with SO_4^- concentrations of up to ~5000 mg L^{-1} to dilute them with DIW into the <200 mg L^{-1} range.

3.4 | IRMS ^{15}N and ^{18}O results

The performance of the Ti(III) method for N and O isotope analysis was evaluated in detail in several ways. We considered the reproducibility and accuracy of the results obtained using nitrate isotopic reference material solutions made in DIW and LNSW and the reproducibility of replicate control standards, and compared the Ti results for selected groundwater samples analyzed by the Cd and bacterial methods.

TABLE 2 Effect of $SO_4^=$ concentration and Ti(III) on the yield of N_2O, and the $\delta^{15}N$ and $\delta^{18}O$ values of nitrate. Mix 1 = $NaHCO_3$ (820 mg L^{-1}) made with degassed deionized water (DIW). Mix 5 = 4000 mgL^{-1} Na_2SO_4 made with degassed DIW. The corresponding mixtures spanned a range of sulfate and bicarbonate concentrations typically encountered in surface, groundwater and seawater. Each mixture had 7.2 mg L^{-1} USGS34, USGS35 or IAEA-KNO_3 added (1 mg L^{-1} NO_3^-). Each subsample was acidified to <pH 2 by adding 0.2 mL 10% HCl. The Ti/sample ratio was 1:20. Means of $n = 2$ measurements shown per sample

Sample	SO_4 [mg L^{-1}]	HCO_3 [mg L^{-1}]	N_2O (nA)	$\delta^{15}N$ USGS34	$\delta^{18}O$ USGS34	$\delta^{15}N$ USGS35	$\delta^{18}O$ USGS35	$\delta^{15}N$ IAEA	$\delta^{18}O$ IAEA
DIW	0	0	25.0	−10.4	43.5	−7.1	122.0	−4.8	92.7
Mix1	0	586	25.6	−10.4	42.6	−7.1	122.4	−4.4	92.5
Mix2	676	439	24.7	−11.4	41.7	−7.3	121.9	−5.0	92.1
Mix3	1351	293	23.4	−11.9	41.2	−7.8	121.0	−5.5	91.3
Mix4	2027	146	23.2	−12.3	41.4	−8.2	120.9	−6.2	90.9
Mix5	2700	0	22.8	−12.2	41.3	−8.3	120.1	−6.5	90.8
		Mean		−11.3	42.0	−7.6	121.4	−5.4	91.7
		Stdev.		0.8	0.9	0.5	0.9	0.8	0.8

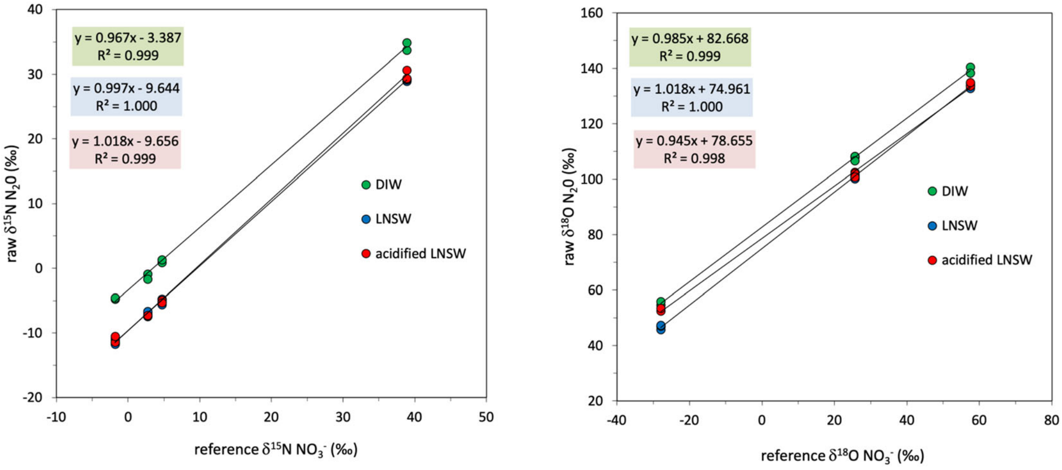

FIGURE 3 Cross plots of reference NO_3^- isotopic ratios vs $\delta^{15}N$ values (left) or $\delta^{18}O$ values (right) of N_2O produced from the Ti(III) reaction at a reagent-to-sample volume of 1:40. Solutions were made up in deionized water (DIW), low nutrient seawater (LNSW) and HCl-acidified (pH 2–3) LNSW [Color figure can be viewed at wileyonlinelibrary.com]

3.5 | Calibrations using nitrate reference solutions

Cross plots of uncorrected $\delta^{15}N$ and $\delta^{18}O$ IRMS values for evolved N_2O using the Ti(III) reaction versus their accepted δ-values gave strong linear relationships, with $r^2 > 0.999$ (Figure 3). In contrast to the Cd-azide method,[28] but similar to the bacterial method,[26] no extraneous N (e.g. from azide) is added to the product N_2O such that the slopes of $\delta^{15}N$ reference calibrations should be unity. Accordingly, our observed slopes of the raw $\delta^{15}N$ values after blank correction vs the known $\delta^{15}N$ values were close to 1 regardless of whether the reference solutions were made up in DIW, LNSW, or HCl-acidified LNSW (pH 2–3). The $\delta^{18}O$ calibration slope could fall below unity if there were O isotope exchange between reaction intermediates, specifically NO_2^- (HNO_2 at acidic pH) and water. However, slopes close to 1 were always observed, suggesting that there was little to no oxygen isotope exchange (see section 3.9) and that the NO_2^- reduction step must be very fast.

Intercept values for the $\delta^{15}N$ calibration would be expected to be close to zero if the N_2O yields were 100% quantitative in accordance with conservation of isotope mass balance ($\delta^{15}N$ value of the N_2O product = $\delta^{15}N$ value of the NO_3^- source). However, we often observed negative intercepts ($\delta^{15}N$ value of the N_2O product < $\delta^{15}N$ value of the NO_3^-) probably from incomplete recovery and N isotope fractionation during branching between reduction pathways leading to N_2O vs NH_4^+ or N_2. The intercept values for the $\delta^{18}O$ calibration were high (74 to 83‰) due to oxygen isotope fractionation preferring ^{16}O during removal of O atoms (5 out of 6). The variability in the proportional yields between these potential pathways and the associated N_2O yield would be expected to degrade the method accuracy and precision for N and O isotopic analyses; however, the high r^2 values and excellent reproducibility that we observed suggested that this was not the case. In addition to variation between DIW and LNSW, variation in the intercepts for both the $\delta^{15}N$ and the $\delta^{18}O$ values occurred as a function of the reagent to sample volume ratio as well as salinity (or sulfate concentration) or NO_3^- concentration, emphasizing that for optimal results, one needs to match samples and standards with respect to sample nitrate concentration, but also the chemical matrix of the samples and references. Fortunately, these can be acceptably controlled, but practitioners need to be aware of these potential (or unforeseen) complications.

3.6 | Mixtures of reference solutions

Gravimetric mixtures of nitrate reference materials were made up using DIW and processed as unknown samples by IRMS to obtain a wide range of $\delta^{15}N$, $\delta^{18}O$ or $\delta^{17}O$ values that reflect a maximal δ-range in nature (Table 3). Mixtures of USGS32 and IAEA-KNO_3 were used to obtain a wide range of $\delta^{15}N$ values in NO_3^- from +4.7 to +180‰. USGS35 and USGS34 were mixed to produce a wide range of $\delta^{18}O$ and $\delta^{17}O$ values in nitrate, spanning +57.5 to −27.9‰ and +51.5 to −14.8‰, respectively. The four endmembers were made up as 71.4 μM (1 mg L^{-1}-N) solutions in degassed DIW which were gravimetrically mixed in ratios of 0:100, 25:75, 50:50, 75:25 and 100:0, and reacted using the IAEA procedures above. The IRMS results for the $\delta^{15}N$ and $\delta^{18}O$ values were accurate and within acceptable analytical uncertainty (mean SD = ±0.52‰ and ±0.74‰, respectively), which was comparable with the analytical uncertainties reported for the Cd or microbial method.

Isotopic assays using the N_2O laser instrument (Table 3) yielded similar uncertainties for $\delta^{15}N$ values to IRMS (SD = ±0.6‰), but the analytical uncertainty was slightly higher for $\delta^{18}O$ values (±1.3‰). The mean uncertainties for $\delta^{17}O$ values were accordingly higher (±2.6‰) owing to the lower ^{17}O content and laser instrument detection limits. Nevertheless, the laser outcomes showed accurate results at acceptable precision for N and O isotopes.

TABLE 3 Results of Ti(III) chloride conversion of nitrate into N_2O using mixtures of USGS32 and IAEA-NO_3 or USGS35 and USGS34 in DIW to obtain a wide range of δ-values and using the laser preparative method. *Italic* denotes gravimetrically estimated nitrate δ-values for $\delta^{15}N_{AIR}$, $\delta^{18}O_{VSMOW}$ and $\delta^{17}O_{VSMOW}$. For all samples $n = 2$ (±SD). For each sample, the same headspace N_2O gas was subsampled for isotopic analysis by CF-IRMS. A reagent-to-sample ratio (v/v) of 1:40 was used [Color table can be viewed at wileyonlinelibrary.com]

Sample	Mixing ratio	$\delta^{15}N_{AIR}$ ‰	$\delta^{18}O_{VSMOW}$ ‰ Gravimetric estimate	$\delta^{17}O_{VSMOW}$ ‰	$\delta^{15}N_{AIR}$ CF-IRMS Measured	$\delta^{18}O_{VSMOW}$ CF-IRMS	$\delta^{15}N^{a}_{AIR}$ laser	$\delta^{18}O_{VSMOW}$ laser	$\delta^{17}O_{VSMOW}$ laser
^{15}N mixture test									
USGS32:IAEA-NO_3	100:0	*+180.0*	-	-	+179.8 ± 0.7	-	+179.3 ± 0.7	-	-
USGS32:IAEA-NO_3	75:25	*+136.1*	-	-	+136.3 ± 0.2	-	+136.9 ± 0.6	-	-
USGS32:IAEA-NO_3	50:50	*+92.3*	-	-	+92.1 ± 0.8	-	+92.8 ± 0.7	-	-
USGS32:IAEA-NO_3	25:75	*+48.7*	-	-	+49.3 ± 0.3	-	+48.5 ± 0.5	-	-
USGS32:IAEA-NO_3	0:100	*+4.7*	-	-	+4.3 ± 0.6	-	+4.5 ± 0.4	-	-
$^{18}O/^{17}O$ mixture test									
USGS35:USGS34	100:0	-	*+57.5*	*+51.5*	-	+57.7 ± 0.3	-	+58.8 ± 1.2	+47.9 ± 2.0
USGS35:USGS34	75:25	-	*+36.2*	*+35.0*	-	+36.7 ± 0.2	-	+35.3 ± 0.9	+36.9 ± 1.0
USGS35:USGS34	50:50	-	*+14.9*	*+18.4*	-	+13.9 ± 0.9	-	+13.8 ± 2.0	+19.7 ± 6.8
USGS35:USGS34	25:75	-	*−6.5*	*+1.8*	-	−6.8 ± 0.4	-	−7.3 ± 1.0	+4.3 ± 2.3
USGS35:USGS34	0:100	-	*−27.9*	*−14.8*	-	−27.2 ± 1.9	-	−26.4 ± 1.6	−16.9 ± 0.7

3.7 | Correction for sample NO_3^- concentration variations

In practice, field sample nitrate concentrations (determined or provided) are not always as accurate as those of gravimetrically prepared isotopic reference solutions relied upon by the isotope laboratory, resulting in variable sample N_2O signals (peak heights) compared with the reference-based target IRMS N_2O signal. As the analyst must have accurate and precise nitrate concentration data for isotopic sample preparations, unreliable nitrate concentrations require a new nitrate assay and/or repeated isotopic analyses when the IRMS N_2O yield falls beyond the acceptable target range (adding cost and time). This aspect of uncontrolled nitrate concentration variance is a perennial issue in isotope laboratories conducting nitrate isotopic assays but is particularly critical for Ti(III) owing to N_2O yield and isotope calibrations that vary with NO_3^- concentration. We used two approaches to overcome this uncontrolled nitrate concentration problem. The first approach was to reject N or O isotopic results for samples that exceeded ±5–10% of the target N_2O yield as determined by nitrate reference calibration solutions. Samples that failed to meet strict N_2O targets were repeated by readjusting the amount of sample in the Ti(III) preparation and remeasured to the correct target N_2O concentration in the repetition.

An alternative approach was to determine and apply an isotopic correction for this titanium-sample-reference (TSR) concentration effect by adding five samples of a nitrate control standard to each autorun, purposely spanning the expected amount of unknown sample nitrate concentration variance. An example of a TSR correction and improvement of results is illustrated in Table 4. To derive the TSR correction, a linear regression of raw $\delta^{15}N$ and $\delta^{18}O$ values (y-axis) vs the IRMS N_2O signal (mV, nA) was obtained from these five samples. The slopes (^{15}m or ^{18}m) were obtained for the ^{15}N and ^{18}O regression lines for the TSR samples. A N_2O signal normalization factor was then applied for all samples (e.g. using 30 nA N_2O target):

$$\text{Normalization factor} = (\text{nA}_{meas} - 30\text{ nA})/30\text{ nA} \qquad (3)$$

Next, the appropriate TSR correction was applied to the N and O isotope data for each sample:

$$\delta^{15}\text{N}(\text{corrected}) = \text{Sample } \delta^{15}\text{N}_{meas} - \left(\text{Norm Factor}^{*}\ {}^{15}m\right) \qquad (4)$$

$$\delta^{18}\text{O}(\text{corrected}) = \text{Sample } \delta^{18}\text{O}_{meas} - \left(\text{Norm Factor}^{*}\ {}^{18}m\right) \qquad (5)$$

Table 4 illustrates the efficacy of this correction to help overcome minor nitrate concentration variances and avoid unnecessary sample repetitions.

3.8 | Low nitrate concentrations and detection limits

To evaluate lower nitrate detection limits for N and O isotopic assays, we conducted Ti(III) conversion and isotope analyses of USGS34, USGS35 and IAEA-KNO_3 and reference materials at 50 ppb NO_3^--N

TABLE 4 Correction for isotopic variance due to changes in the TSR using a 1:20 reagent-to-sample volume ratio for lab control standard KNO_3. The regression slopes (*m*) for $\delta^{15}N$ and $\delta^{18}O$ values on this dataset were 5.29 and 6.10, respectively (see Equations 3–5). The precision of the δ-results was significantly improved to accommodate a 20% range of unexpected nitrate variance and yielded acceptable uncertainties for $\delta^{15}N$ and $\delta^{18}O$ values of ±0.5 and ±0.3 permil, respectively. nA = N_2O signal with IRMS [Color table can be viewed at wileyonlinelibrary.com]

NO_3 mgL^{-1}	nA	$\delta^{15}N$ (meas.)	$\delta^{18}O$ (meas.)	(norm. Factor)	$\delta^{15}N$ corr.	$\delta^{18}O$ corr.
0.8	21.7	−7.5	90.4	−0.3	−5.8	91.9
0.8	23.4	−8.5	90.2	−0.2	−7.2	91.4
0.9	26.8	−6.8	91.4	−0.1	−6.2	92.0
0.9	26.7	−6.6	91.0	−0.1	−6.0	91.6
1.0	30.5	−6.7	91.5	0.0	−6.8	91.4
1.0	30.1	−6.3	91.3	0.0	−6.3	91.3
1.1	31.6	−5.9	92.2	0.1	−6.2	91.9
1.1	30.2	−5.4	92.4	0.0	−5.4	92.4
1.2	33.9	−5.8	92.4	0.1	−6.6	91.7
1.2	34.4	−5.4	92.3	0.1	−6.3	91.6
Mean		**−6.5**	**91.5**		**−6.3**	**91.7**
SD		**1.0**	**0.8**		**0.5**	**0.3**

(3.5 µM) on the Isoprime 100 at the IAEA (not shown). The mean N_2O IRMS signal was 6.7 nA compared with 30 nA for our routine analysis and a blank of 0.8 nA. The results were accurate with acceptable uncertainties of ±0.6 for both $\delta^{15}N$ and $\delta^{18}O$ values. It could be feasible to obtain $\delta^{15}N$ and $\delta^{18}O$ values possibly to 10 ppb (0.7 µM) by increasing the IRMS ion source sensitivity and by pre-evacuating the sample vials to eliminate the blank; however, this aspect of further lowering detection limits requires more testing.

3.9 | Oxygen isotope exchange

The observed $\delta^{18}O$ calibration slopes close to 1 implied little oxygen isotope exchange with water during the Ti(III) reduction of NO_3^- to N_2O. Because of the ability to measure ^{17}O, we further tested for oxygen isotope exchange using the N_2O laser. A KNO_3 laboratory standard was dissolved (at 71.4 µM or 1 mg L^{-1} N) in nitrate-free tap water having $\delta^{18}O$ values adjusted from −10.2 to +22.0‰ (VSMOW) and an electrical conductivity of ~425 µS/cm. The water $\delta^{18}O$ values were adjusted from their initial value ($\delta^{18}O$ = −10.2‰, $\delta^{17}O$ = −5.3‰) by gravimetrically adding small amounts of 97.0 atom % ^{18}O water (Sigma Aldrich P/N 329878: the ^{17}O content of the ^{18}O-enriched water was unknown) to 500 g of tap water. The results from laser spectroscopic analysis showed that, despite the 33‰ range in the $\delta^{18}O$ values (we presume that the ^{17}O of water varied accordingly) of water of the dissolved NO_3^- samples, there was no effect of water oxygen on either the $\delta^{18}O$ or the $\delta^{17}O$ values of the N_2O produced from nitrate through the Ti reaction (Table 5).

TABLE 5 Test for oxygen isotope exchange during the conversion of 0.2 mg L^{-1} NO_3^--N (KNO_3 laboratory standard) by Ti(III) chloride into N_2O for isotopic analysis by laser spectroscopy using a ratio of 1:20. The $\delta^{18}O_{VSMOW}$ values of the water used to dissolve the KNO_3 ranged by 33‰. *n* = 4 (±SD) for uncorrected oxygen δ -values for N_2O from the laser instrument

Water $\delta^{18}O_{VSMOW}$	$\delta^{18}O$-N_2O	$\delta^{17}O$-N_2O	Sample
−10.2 ‰	+107.6 ± 0.5 ‰	+59.3 ± 0.4 ‰	IHL-NO_3
+7.9 ‰	+107.1 ± 0.3 ‰	+59.7 ± 1.5 ‰	IHL-NO_3
+22.9 ‰	+107.2 ± 0.5 ‰	+58.4 ± 0.5 ‰	IHL-NO_3

3.10 | Comparison of the Ti method vs the microbial and Cd method

For a methodological comparison test, a selection of nitrate-contaminated groundwater samples from IAEA projects in South and Central America (Argentina, Bolivia, Dominican Republic, Chile, Mexico), which had been previously measured for their $\delta^{15}N$ and $\delta^{18}O$ values using the microbial method at the University of Calgary (Calgary, Canada), were re-measured using the Ti(III) method and the Cd-azide method at the IAEA laboratory. These samples had been stored frozen at the IAEA for more than 1.5 years before remeasuring by Cd and Ti(III). This test was undertaken as a preliminary assessment to determine how these different methods compared with each other for the analysis of uncontrolled environmental samples. These samples had NO_3^- concentrations ranging between 0.2 and 87 mg L^{-1} NO_3-N (14–6100 µM), with electrical conductivities ranging from 129 to 5340 µS/cm. No $SO_4^=$ concentrations were provided, and the nitrate concentrations provided by the client were frequently inaccurate when the samples were re-tested at the IAEA. Hence, these unknown samples represented a worst-case scenario for a laboratory. The Ti method for these samples was conducted using a 1:20 reagent ratio using the IRMS SOP.

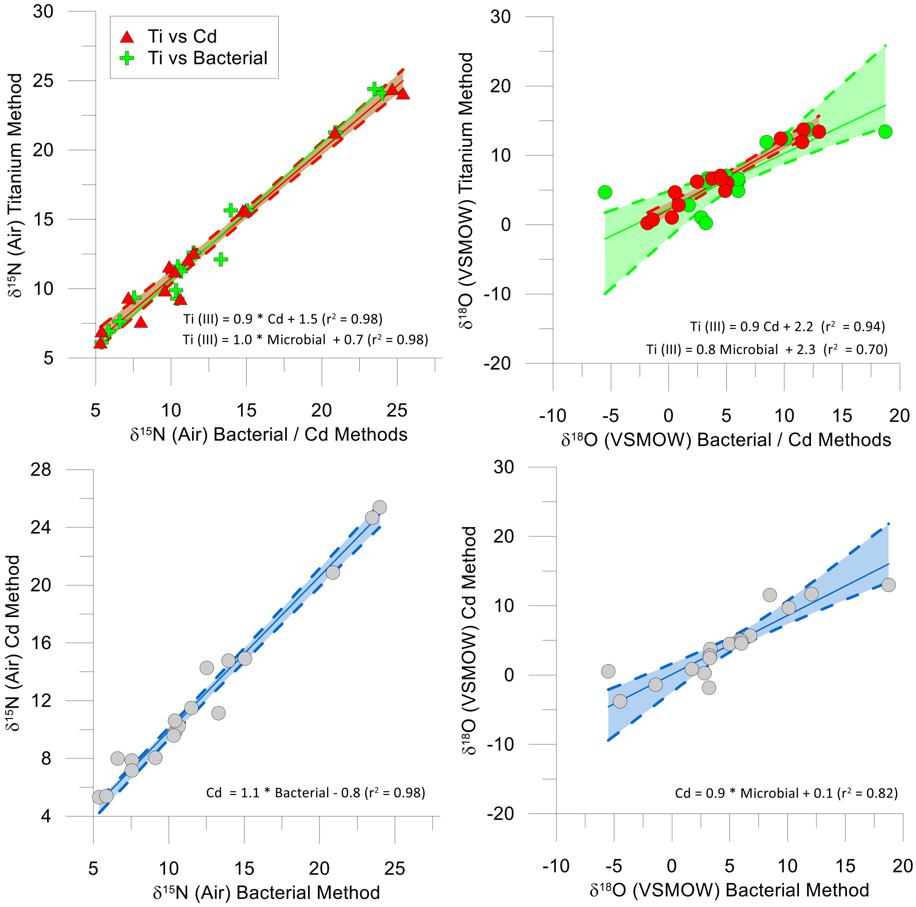

FIGURE 4 Comparison of δ^{15}N and δ^{18}O values of nitrate from archived groundwater samples using Ti(III) (1:20) versus the denitrifier and Cd-azide method, and bacterial versus Cd-azide method. Samples were initially run by the bacterial method at the University of Calgary, then returned and stored frozen at the IAEA for 1.5 years [Color figure can be viewed at wileyonlinelibrary.com]

The methodological comparative results for archived ground water samples are summarized in Figure 4. For δ^{15}N values, the calibrated values determined using the Ti method plotted against the Cd and microbial methods agreed well, and for all methods the control standards gave accurate results. The slopes were close to unity ($r^2 > 0.9$) against the Cd and microbial methods. For δ^{18}O values, the comparison was not quite as good. The best comparative result for the Ti(III) method was versus the Cd method; this regression yielded a slope of 0.9 ($r^2 = 0.94$). The microbial comparison was considerably worse with a slope of 0.8 ($r^2 = 0.70$). Correspondingly, the comparative results of the Cd-azide versus microbial method were similar (Figure 4). The intercepts were variable between the N_2O preparative methods but, in the case of the δ^{18}O values, heavily influenced by outlier points. Given that the Cd and Ti methods, in general, compared well, it is suggested there can be systematic differences possibly due to sample chemistry that affected the δ^{18}O values of the microbial method. No complete water chemistry (e.g. cations or anions) was provided with these samples other than nitrate and electrical conductivity; it remains unknown if any of the samples had excessive $SO_4^{=}$ interferences affecting the Ti method for δ^{18}O values. Finally, this comparative test suggests, despite the limitations, that systematic international inter-comparison and proficiency testing for the stable isotopes of nitrates using different preparation method and instrumentation is warranted and overdue.

4 | CONCLUSIONS

Our new Ti(III) method for δ^{15}N, δ^{18}O and δ^{17}O values compares favorably with existing methods for the preparation of water samples for NO_3^- isotopic determination. With respect to minimal effort and low cost per sample, the method excels in comparison with the microbial and Cd-azide methods. The preparation time is only a few minutes per sample and the handling time per sample is 1–2 days including isotope analysis. Assuming that vials are reused, the material cost up to the point of IRMS or laser analysis is < $2USD for a small amount of reagent and vial septa. The precision and reproducibility of the δ^{15}N and δ^{18}O values using either IRMS or laser analysis are comparable with literature values for the microbial or Cd-azide method. The δ^{17}O results using laser analysis were similar to or better than those from N_2O conversions into O_2. The calibration plots have high r^2 values with slopes near the theoretical value of 1 after blank correction. For environmental samples, a reasonable comparison was obtained with results produced by the microbial and Cd-azide methods; however, given the uncertainties in storage time and lack of water chemistry, a properly controlled nitrate isotope inter-comparison of various N_2O conversion methods is clearly warranted.

The main caveat of the Ti(III) method, however, is the non-quantitative yield of N_2O resulting in isotopic discrimination and negative intercepts for the calibration cross plots. Nevertheless, the N_2O yields are typically >65% such that analytical sensitivity is minimally impacted. In addition, unexpected variance in sample concentration of NO_3^- and ions such as sulfate cause variations in N_2O yield and apparent isotope discrimination. However, as we have in, these factors can be controlled for when reference solutions are matched to samples. The precision and accuracy are within literature values for the microbial and Cd-azide methods. Given the quality of results, ease of use and relatively low cost, the Ti(III) reduction of NO_3^- to N_2O is a useful and simpler alternative to existing preparation methods for NO_3^- isotope analysis.

4.1 | Future research

The chemistry of the Ti(III) reduction of NO_3^- and NO_2^- to N_2O has been previously investigated and the major intermediates and the kinetics of each reductive step are reasonably well known.[32] It is also known that the end products include N_2O, N_2 and NH_4^+, verifying our observations that N_2O is a major product at low pH. Hence, we surmise that variations in N_2O yield with variation in sample chemistry are due to changes in reaction kinetics in favor of either N_2 or NH_4^+ as end products. It remains unclear how these conditions affect reaction pathways and end-product yield, suggesting that future work is warranted to improve or perfect the Ti(III) method by identifying and adopting conditions that can ensure 100% yield of N_2O over the range in composition of environmental samples. We acknowledge that some knowledge gaps remain, particularly concerning the Ti(III) nitrate into N_2O conversion stoichiometry and intermediates. For example, we do not know whether high concentrations of dissolved organic carbon (DOC), for example, in soil porewater leachate would impact the Ti(III) conversion into N_2O. However, we anticipate that future research and efforts may provide insights possibly leading to quantitative conversions of nitrate into N_2O.

ACKNOWLEDGEMENTS

This work was funded by the University of Massachusetts at Dartmouth, USA, and the International Atomic Energy Agency in Vienna, Austria.

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REFERENCES

1. Vitousek PM, Aber JD, Howarth RW, et al. Human alteration of the global nitrogen cycle: Sources and consequences. *Ecol Appl*. 1997; 7:737-750.
2. Galloway JN, Aber JD, Erisman JW, et al. The nitrogen cascade. *Bioscience*. 2003;53(4):341-356.
3. Gruber N, Galloway JN. An earth-system perspective of the global nitrogen cycle. *Nature*. 2008;451(7176):293-296.
4. Diaz RJ, Rosenberg R. Spreading dead zones and consequences for marine ecosystems. *Science*. 2008;321(5891):926-929.
5. Bouchard DC, Williams MK, Surampalli RY. Nitrate contamination of groundwater - sources and potential health-effects. *J AWWA*. 1992; 84(9):85-90.
6. Randall GW, Mulla DJ. Nitrate nitrogen in surface waters as influenced by climatic conditions and agricultural practices. *J Environ Qual*. 2001;30(2):337-344.
7. Power JF, Schepers JS. Nitrate contamination of groundwater in North America. *Agr Ecosyst Environ*. 1989;26(3-4):165-187.
8. Aravena R, Robertson WD. Use of multiple isotope tracers to evaluate denitrification in ground water: Study of nitrate from a large-flux septic system plume. *Ground Water*. 1998;36(6):975-982.
9. Böhlke J, Denver J. Combined use of groundwater dating, chemical, and isotopic analyses to resolve the history and fate of nitrate contamination in two agricultural watersheds, Atlantic coastal plain, Maryland. *Water Resour Res*. 1995;31(9):2319-2339.
10. Sigman D, Altabet M, McCorkle D, Francois R, Fischer G. The $\delta^{15}N$ of nitrate in the Southern Ocean: Consumption of nitrate in surface waters. *Global Biogeochem Cycles*. 1999;13(4):1149-1166.
11. Archana A, Thibodeau B, Geeraert N, Xu MN, Kao SJ, Baker DM. Nitrogen sources and cycling revealed by dual isotopes of nitrate in a complex urbanized environment. *Water Res*. 2018;142:459-470.
12. Puig R, Soler A, Widory D, Mas-Pla J, Domenech C, Otero N. Characterizing sources and natural attenuation of nitrate contamination in the Baix Ter aquifer system (NE Spain) using a multi-isotope approach. *Sci Total Environ*. 2017;580:518-532.
13. Amberger A, Schmidt HL. Natürliche isotopengehalte von Nitrat als Indikatoren für dessen Herkunft. *Geochim Cosmochim Acta*. 1987; 51(10):2699-2705.
14. Wassenaar LI. Evaluation of the origin and fate of nitrate in the Abbotsford aquifer using the isotopes of ^{15}N and ^{18}O in NO_3. *Appl Geochem*. 1995;10(4):391-405.
15. Suchy M, Wassenaar LI, Graham G, Zebarth B. High-frequency NO_3^- isotope ($\delta^{15}N$, $\delta^{18}O$) patterns in groundwater recharge reveal that short-term land use and climatic changes influence nitrate contamination. *Hydrol Earth Syst Sci*. 2018;22(8):4267-4279.
16. Mariotti A, Germon JC, Hubert P, et al. Experimental determination of nitrogen kinetic isotope fractionation: Some principles; illustration for the denitrification and nitrification processes. *Plant and Soil*. 1981;62(3):413-430.
17. Cline J, Kaplan I. Isotopic fractionation of dissolved nitrate during denitrification in the eastern tropical North Pacific Ocean. *Mar Chem*. 1975;3(4):271-299.
18. Granger J, Sigman DM, Gagnon J, Tremblay JE, Mucci A. On the properties of the Arctic halocline and deep-water masses of the Canada Basin from nitrate isotope ratios. *J Geophys Res Oceans*. 2018;123(8):5443-5458.
19. Rafter PA, Sigman DM. Spatial distribution and temporal variation of nitrate nitrogen and oxygen isotopes in the upper equatorial Pacific Ocean. *Limnol Oceanogr*. 2016;61(1):14-31.
20. Gebus B, Halas S. *Isotopic studies of nitrate - a review*. Poland: Annales Universitatis Mariae Curie; 2015.
21. Bremner JM, Keeney DR. Steam distillation methods for determination of ammonium, nitrate and nitrite. *Anal Chim Acta*. 1965;32:485-495.

22. Silva SR, Kendall C, Wilkison DH, Ziegler AC, Chang CC, Avanzino RJ. A new method for collection of nitrate from fresh water and the analysis of nitrogen and oxygen isotope ratios. *J Hydrol*. 2000; 228(1-2):22-36.
23. Sigman D, Altabet M, Michener R, McCorkle D, Fry B, Holmes RJMC. Natural abundance-level measurement of the nitrogen isotopic composition of oceanic nitrate: An adaptation of the ammonia diffusion method. *Mar Chem*. 1997;57(3-4):227-242.
24. McIlvin MR, Casciotti KL. Technical updates to the bacterial method for nitrate isotopic analyses. *Anal Chem*. 2011;83(5):1850-1856.
25. Casciotti KL, Sigman DM, Hastings MG, Böhlke JK, Hilkert A. Measurement of the oxygen isotopic composition of nitrate in seawater and freshwater using the denitrifier method. *Anal Chem*. 2002;74(19):4905-4912.
26. Sigman DM, Casciotti K, Andreani M, Barford C, Galanter M, Bohlke JK. A bacterial method for the nitrogen isotopic analysis of nitrate in seawater and freshwater. *Anal Chem*. 2001;73(17):4145-4153.
27. Soto DX, Koehler G, Hobson KA. Combining denitrifying bacteria and laser spectroscopy for isotopic analyses (δ^{15}N, δ^{18}O) of dissolved nitrate. *Anal Chem*. 2015;87(14):7000-7005.
28. McIlvin MR, Altabet MA. Chemical conversion of nitrate and nitrite to nitrous oxide for nitrogen and oxygen isotopic analysis in freshwater and seawater. *Anal Chem*. 2005;77(17):5589-5595.
29. Tu Y, Fang Y, Liu D, Pan Y. Modifications to the azide method for nitrate isotope analysis. *Rapid Commun Mass Spectrom*. 2016;30(10): 1213-1222.
30. Wassenaar LI, Douence C, Altabet MA, Aggarwal PK. N and O isotope ($\delta^{15}N^{\alpha}$, $\delta^{15}N^{\beta}$, δ^{18}O, δ^{17}O) analyses of dissolved NO_3- and NO_2- by the Cd-azide reduction method and N_2O laser spectrometry. *Rapid Commun Mass Spectrom*. 2018;32(3):184-194.
31. Kaiser J, Hastings MG, Houlton BZ, Rockmann T, Sigman DM. Triple oxygen isotope analysis of nitrate using the denitrifier method and thermal decomposition of N_2O. *Anal Chem*. 2007;79(2):599-607.
32. Goldstein S, Behar D, Rajh T, Rabani J. Nitrite reduction to nitrous oxide and ammonia by TiO_2 electrons in a colloid solution via consecutive one-electron transfer reactions. *J Phys Chem a*. 2016; 120(15):2307-2312.
33. Al-Wehaid A, Townshend A. Spectrophotometric flow-injection determination of nitrate based on reduction with titanium (iii) chloride. *Anal Chim Acta*. 1986;186:289-294.
34. Nakata R, Terashita M, Nitta A, Ishikawa K. Spectrophotometric or coulometric determination of nitrate with an electrochemical reductor using flow injection. *Analyst*. 1990;115(4):425-430.
35. Cho S-J, Sasaki S, Ikebukuro K, Karube I. A simple nitrate sensor system using titanium trichloride and an ammonium electrode. *Sens Actuators B*. 2002;85(1-2):120-125.
36. Foreman RK, Segura-Noguera M, Karl DM. Validation of Ti (III) as a reducing agent in the chemiluminescent determination of nitrate and nitrite in seawater. *Mar Chem*. 2016;186:83-89.
37. Cho S. A fluorescent nitrate sensing system using a reaction cartridge and titanium trichloride. *Talanta*. 2001;54(5):903-911.
38. Burns EA. Reduction of ammonium nitrate with titanium (III) chloride in acid media. *Anal Chim Acta*. 1962;26:143-147.
39. Rich DW, Grigg B, Snyder GH. Determining ammonium and nitrate using a gas sensing ammonia electrode. *Soil Crop Sci Soc Fla*. 2006; 65:1-4.
40. Ashton JF. Some aspects of the solution chemistry of titanium (III). Masters thesis, University of Tasmania (Hobart); 1977.
41. Bohlke JK, Mroczkowski SJ, Coplen TB. Oxygen isotopes in nitrate: New reference materials for $^{18}O:^{17}O:^{16}O$ measurements and observations on nitrate-water equilibration. *Rapid Commun Mass Spectrom*. 2003;17(16):1835-1846.
42. Savard MM, Cole AS, Vet R, Smirnoff A. The Δ^{17}O and δ^{18}O values of atmospheric nitrates simultaneously collected downwind of anthropogenic sources–implications for polluted air masses. *Atmos Chem Phys*. 2018;18(14):10373-10389.
43. Granger J, Sigman DM. Removal of nitrite with sulfamic acid for nitrate N and O isotope analysis with the denitrifier method. *Rapid Commun Mass Spectrom*. 2009;23:753-3762.
44. Bohlke JK, Gwinn CJ, Coplen TB. New reference materials for nitrogen-isotope-ratio measurements. *Geostand Geoanal Res*. 1993; 17(1):159-164.
45. Brand WA, Coplen TB, Vogl J, Rosner M, Prohaska T. Assessment of international reference materials for isotope-ratio analysis (IUPAC technical report). *Pure Appl Chem*. 2014;86(3):425-467.
46. Snider DM, Venkiteswaran JJ, Schiff SL, Spoelstra J. From the ground up: Global nitrous oxide sources are constrained by stable isotope values. *PLoS ONE*. 2015;10(3):e0118954.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Altabet MA, Wassenaar LI, Douence C, Roy R. A Ti(III) reduction method for one-step conversion of seawater and freshwater nitrate into N_2O for stable isotopic analysis of $^{15}N/^{14}N$, $^{18}O/^{16}O$ and $^{17}O/^{16}O$. *Rapid Commun Mass Spectrom*. 2019;33:1227–1239. https://doi.org/10.1002/rcm.8454