

# Exploring the potential of Electrospray-Orbitrap for stable isotope analysis using nitrate as a model

Andreas Hilkert<sup>1,\*</sup>, John K. Böhlke<sup>2</sup>, Stanley J. Mroczkowski<sup>2</sup>, Kyle L. Fort<sup>1</sup>, Konstantin Aizikov<sup>1</sup>, Xingchen T. Wang<sup>3</sup>, Sebastian H. Kopf<sup>4,5</sup>, Cajetan Neubauer<sup>5,\*</sup>

1. Thermo Fisher Scientific (Bremen), Hanna-Kunath Strasse 11, 28199 Bremen, Germany.
2. U.S. Geological Survey, Reston, Virginia 20192, USA.
3. Department of Earth and Environmental Sciences, Boston College, Chestnut Hill, MA 02467, USA.
4. Department of Geological Sciences, University of Colorado, Boulder, Colorado 80309, USA.
5. Institute of Arctic and Alpine Research, University of Colorado, Boulder, Colorado 80303, USA.

\* Corresponding authors: [andreas.hilkert@thermofisher.com](mailto:andreas.hilkert@thermofisher.com) (A.H.), [123caj@gmail.com](mailto:123caj@gmail.com) (C.N.)

## Abstract

Widely used isotope ratio mass spectrometers (IRMS) have limited capabilities to measure metabolites, drugs or small polyatomic ions without the loss of structural isotopic information. A new approach has recently been introduced that uses electrospray ionization Orbitrap to measure multi-dimensional isotope signatures of intact polar compounds. Using nitrate as a reference compound, this study aims to establish performance metrics for comparisons with conventional IRMS at the natural abundance-level. We present a framework on how to convert isotopolog intensities to  $\delta$ -values that are commonly used in the isotope geochemistry community. The quantification of seven nitrate isotopologs provides multiple pathways for obtaining the primary N and O  $\delta$ -values including non-mass-dependent O isotope variations, as well as opportunities to explore non-random isotopic distributions (i.e., clumping effects) within molecular nitrate. Using automation and the adaptation of measurement principles that are specific to isotope ratio analysis, nitrate  $\delta^{15}\text{N}_{\text{AIR}}$ ,  $\delta^{18}\text{O}_{\text{VSMOW}}$  and  $\delta^{17}\text{O}_{\text{VSMOW}}$  were measured with a long-term precision of 0.4 ‰ or better for isotopic reference materials and purified nitrate from environmental samples. In addition we demonstrate promising results for unpurified environmental samples in liquid form. With these new developments, this study connects the two largely disparate mass spectrometry fields of bioanalytical MS and isotope ratio MS, thus providing a route to measure new isotopic signatures in diverse organic and inorganic solutes.

**Keywords:** Isotopologue, isotopolog, isotope ratio mass spectrometry, polar compounds, oxyanions, metabolomics, clumped isotopes

## Introduction

Stable isotope variations allow unique insights into natural processes, because they can be used to relate actions that take place at the atomic level to effects on much larger scales. Typically, stable isotope ratios of polar solutes are measured after the analyte has been converted into a low molecular weight gas by combustion, pyrolysis, microbial fermentation, or other chemical treatments. The gas is then examined on a magnetic sector isotope ratio mass spectrometer (IRMS).<sup>1</sup> However, in these methods much of the intra-molecular isotopic information is erased during the formation of gases and by destructive ionization in the IRMS. Despite complementary techniques,<sup>2,3</sup> a significantly different and more sensitive approach is needed to access the native isotopic “fingerprints” that are recorded by intact molecules. Such patterns have long been thought to be potentially useful tools to elucidate processes and mechanisms that shape the functioning of cells and ecosystems.<sup>4</sup>

Liquid chromatography mass spectrometry (LC/MS) studies performed over the last decade point to a plausible path via soft ionization techniques such as electrospray ionization (ESI).<sup>5–9</sup> Recent work on unlabeled amino acids and oxyanions shows that in particular ESI coupled to a quadrupole-Orbitrap analyzer (ESI-Orbitrap) is a promising technology for analyzing the isotopic information of intact polar compounds at natural-abundance level.<sup>10–13</sup> However, important gaps exist between these proof-of-concept studies and a method suitable for routine usage. For example, a framework for converting the isotopolog intensities to  $\delta$ -values that are commonly reported in the isotope geochemistry community has yet to be established. In addition, previous work has relied on manual injection of samples, limiting throughput and precision (i.e., related to human errors or interruption of the spray). In this study, we improve ESI-Orbitrap-based isotopic spectrometry for analyzing natural-abundance isotopic composition of intact polar compounds and compare it to well-established IRMS techniques.

Specifically, we use nitrate as a reference model in this study. Nitrate ( $\text{NO}_3^-$ ) is one of the simplest polar compounds and it is a key component of the global cycling of nitrogen in natural and human-impacted environments.<sup>14,15</sup> The biogeochemical research community provides a firm foundation for the analysis and interpretation of nitrate isotopes. Optimized methods<sup>16-19</sup> and a suite of calibrated international reference materials<sup>20-22</sup> make nitrate our preferred model for developing isotope ratio measurements by ESI. The relative abundances of the three heavier isotopes in nitrate ( $^{15}\text{N}$ ,  $^{18}\text{O}$ ,  $^{17}\text{O}$ ) in comparison to the more abundant, lower mass isotopes ( $^{14}\text{N}$ ,  $^{16}\text{O}$ ) reveal how nitrogen moves among living and non-living forms in the biosphere and geosphere. For example, the isotope ratios can reveal microbial pathways of nitrification and denitrification, as well as sources and fate of natural and anthropogenic nitrogen inputs to the environment.<sup>23,24</sup>

In this paper, we report measurement principles that are designed for automated and reproducible analysis of isotopes in intact molecules by ESI-Orbitrap, with application to nitrate. This is achieved by (1) reconfiguring and fine tuning of technologies that are routinely used as mass spectrometers in metabolomics with the aim of enabling their use for isotope ratio analysis; (2) combining measurements performed separately with and without the dominant isotopolog peak; (3) calibrating data with respect to nitrate isotopic reference materials; and (4) testing the method with different types of sample solutions of varying complexity including diluted brines and groundwater samples. The focused study of nitrate provides a significant increment of technical advance for isotopic spectrometry by ESI-Orbitrap with approximately 10-fold improvement of sample throughput, sensitivity, isotopic accuracy, as well as bringing the technology to environmental samples.<sup>12</sup> The replication and long-term accuracy of these analyses show that the ESI technology is approaching the stage of practical applications with capabilities not realized by previous methods, including comprehensive molecular isotopic characterization, small sample sizes, and simple pre-treatment in some cases.

## Experimental Section

**Reference materials and environmental samples.** Isotopic reference materials were provided by the United States Geological Survey (USGS; Reston, VA). USGS32, USGS34 and RSIL-N11 (N11) were provided as  $\text{KNO}_3$  salts, USGS35 as  $\text{NaNO}_3$  salt.<sup>18,21,22</sup> Environmental samples archived from previous studies included (1) concentrated brines and soil leachate solutions from desert salt accumulations in representative arid regions on Earth,<sup>25,26</sup> and (2) fresh groundwater samples from different regions in the USA (see Table S2).<sup>26-28</sup> Desert soil leachates were prepared by mixing dry soil with deionized water and filtering.<sup>25,26</sup> Groundwater samples were collected from wells, filtered, and stored cold or with hydroxide as preservative (pH 11-12). The groundwater samples were typical of aquifers affected by agriculture or urban land use, and had relatively high  $\text{NO}_3^-$  concentrations (500-1300  $\mu\text{M}$ ) with varying proportions of  $\text{Cl}^-$ ,  $\text{SO}_4^{2-}$ , and dissolved inorganic carbon, as described elsewhere.<sup>26-28</sup> For selected samples,  $\text{NO}_3^-$  was isolated from mixed salt solutions by trapping on large-volume AG1X8 ion-exchange resin columns, followed by gradual elution with 0.5 M KCl to separate anions.<sup>29</sup> The KCl- $\text{KNO}_3$  eluents, as well as some of the reference materials, were passed through AG-MP50 resin columns in the Ag form to remove Cl and exchange K for Ag, then freeze dried to produce  $\text{AgNO}_3$  salts.<sup>26</sup>

For ESI analysis, solid samples of reference materials and purified environmental nitrate samples were prepared first as 100 mM stock solutions in a 1:1 mixture of LC/MS grade water and methanol. Aliquots of the stock solutions were then diluted to 50  $\mu\text{M}$  with LC/MS grade methanol for isotopic analysis. For “dilute and shoot” analysis of environmental samples, the original aqueous leachates, brines and groundwater samples were diluted to 1  $\mu\text{M}$  nitrate with LC/MS grade methanol and analyzed directly by ESI. For a list of nitrate isotopologs, relative abundances, representative mass spectra, and related definitions see Table 1 and Figure 1.

**MS instrumentation.** For all the analyses, we used a Q Exactive HF Orbitrap (Thermo Fisher Scientific) with an Ion Max API source, which was connected to a HESI-II probe (Thermo Fisher

Scientific) with a low flow needle for flow rates of 1-10  $\mu\text{L}/\text{min}$ . The following ionization settings were used: sheath gas flow rate: 2, auxiliary gas flow rate: 0, sweep gas flow rate: 0, spray voltage:  $\sim 2.7$  kV (negative ionization mode), spray current (observed):  $< 0.2$   $\mu\text{A}$ , capillary temperature:  $275$   $^{\circ}\text{C}$ , S-lens RF level: 70, auxiliary gas heater temp (actual):  $40$   $^{\circ}\text{C}$ .

To maximize the range of measurements that could be done, two different types of scans were performed, one of which included the predominant monoisotopic base peak consisting of only the light isotopes (“with  $M0$ ”) and one of which excluded the base peak so other minor peaks could be resolved more efficiently (“without  $M0$ ”). The following mass spectrometer settings were used. Scan type: selected ion monitoring (SIM); scan ranges (isolation range):  $m/z$  61.2 - 66 (“with  $M0$ ”) and  $m/z$  62.2 - 67 (“without  $M0$ ”); resolution:  $R = 15,000$  or  $30,000$ ; polarity: negative; microscans: 5; lock masses: off; automatic gain control (AGC) target: 300,000; AGC prescan mode: -1; maximum injection time: 1000 ms. Profile mode data were collected using the Q Exactive HF Tune software. The Xcalibur software (Thermo Fisher) was used for data acquisition by flow injection and automation of the dual inlet.

For most tests we chose a nitrate concentration of  $50$   $\mu\text{M}$ , because this concentration did not limit the scan rate of the Orbitrap mass analyzer in the “without  $M0$ ” experiments. The observed injection time was typically  $0.4$  ms in the “with  $M0$ ” experiment and  $25$  ms in the “without  $M0$ ” experiment when using  $50$   $\mu\text{M}$  nitrate solutions (AGC target 300,000). The “dilute and shoot” of  $1$   $\mu\text{M}$  solutions resulted in  $4 - 7$  ms injection time in the “with  $M0$ ” experiment. The maximum Orbitrap scan rate is approximately  $24$  Hz at  $R = 15,000$  and  $14$  Hz at  $R = 30,000$  (at  $m/z$  200).

**Flow injection.** An UltiMate 3000 RSLCnano HPLC system (Thermo Fisher Scientific) was coupled to the Q Exactive HF Orbitrap for “flow injection” of nitrate solutions. The HPLC loading pump was used to carry LC/MS grade methanol at a flow rate of  $4$   $\mu\text{L}/\text{min}$ . The 6-port valve of the autosampler was equipped with a  $20$   $\mu\text{L}$  loop for flow injection resulting in a  $6$  min wide plateau peak. The total analysis time per sample was set to  $15$  min to prevent cross contamination.

Sequences were set up in alternating blocks of 4 injections each for reference and for samples to improve precision and to allow drift correction. A sequence was started always with a block for conditioning. At the end of each block an injection of 1:1 (v/v) HPLC grade methanol:water was used to maintain a stable spray.

**Dual syringe pump ('Dual Inlet').** To enable the continuous delivery of a reference and a sample in an alternating mode, a digitally controlled syringe pump (Fusion 100, Chemyx) loaded with two 500  $\mu$ L HPLC autosampler syringes (gauge22, flat tip; Thermo Fisher Scientific) was coupled to a 6-port valve (Rheodyne). Reference and sample were pumped simultaneously at a flow rate of 4  $\mu$ L/min. After conditioning the system for at least 10 min, reference/sample/reference comparisons were carried out for 30 min or 70 min by switching the valve every 10 min.

**Data processing.** A custom built FTStatistic software module of the Thermo Fisher Scientific data processing software suite was used for the unsupervised extraction of ion intensities from RAW files. The software is available upon request from A. Hilkert. R (version 3.6.3), RStudio, and the R-packages dplyr, tidyr, and ggplot2 were used for data analysis. Final evaluation and calculation of  $\delta$ -values was performed in Microsoft Excel. An example analysis is provided in the SI.

**Calculation of  $\delta$ -values.** Delta ( $\delta$ ) is defined as  $\delta_{sa/STD} = R_{sa}/R_{STD} - 1$  or  $[R_{sa} - R_{STD}]/R_{STD}$ , where *sa* represents a sample, *STD* represents the primary standard, and *R* is the isotope abundance ratio ( $^{15}\text{N}/^{14}\text{N}$ ,  $^{18}\text{O}/^{16}\text{O}$ ,  $^{17}\text{O}/^{16}\text{O}$ ). Nitrate isotopic compositions were calculated and reported relative to the international standards, atmospheric  $\text{N}_2$  nitrogen (AIR) and Vienna Standard Mean Ocean Water oxygen (VSMOW).

Isotopolog abundance ratios of samples were measured against those of a reference solution of a working nitrate laboratory standard RSIL-N11 (N11; see Table S2 in SI). Provisional  $\delta^{15}\text{N}$  values of a sample ("sa") relative to the N11 lab standard were obtained using isotopolog signals measured by ESI in experiments "with M0", for example:

$$\delta^{15}N_{sa/N11} = \frac{(^{15}N^{16}O_3/^{14}N^{16}O_3)_{sample}}{(^{15}N^{16}O_3/^{14}N^{16}O_3)_{N11}} - 1 \quad (1)$$

These values were then expressed relative to the  $\delta^{15}N_{AIR}$  scale using the independently known value of  $\delta^{15}N_{N11/AIR}$  (1-point calibration):

$$\delta^{15}N_{sa/AIR(1)} = \delta^{15}N_{sa/N11} + \delta^{15}N_{N11/AIR} + \delta^{15}N_{sa/N11} \cdot \delta^{15}N_{N11/AIR} \quad (2)$$

The accuracy of the results was tested and improved in some experiments using additional reference samples (e.g., USGS32, USGS34) with much higher or lower  $\delta$ -values than those of N11 to adjust for  $\delta$ -scaling effects (2-point calibration):

$$\delta^{15}N_{sa/AIR(2)} = \delta^{15}N_{RM1/AIR} + \left[ \delta^{15}N_{sa/labstd} - \delta^{15}N_{RM1/labstd} \right]_{meas} \cdot \left[ \delta^{15}N_{RM2/AIR} - \delta^{15}N_{RM1/AIR} \right] / \left[ \delta^{15}N_{RM2/labstd} - \delta^{15}N_{RM1/labstd} \right]_{meas} \quad (3),$$

where *RM1* and *RM2* are nitrate isotopic reference materials with contrasting values (e.g., N11, USGS32, USGS34); and *labstd* is an arbitrary reference material analyzed repeatedly in a given sample set to monitor machine performance and drift. For experiments in the current study, we used N11 as a substitute for both *labstd* and *RM1* in Equation 3. Analogous formulas to Equations 1 to 3 were used to obtain  $\delta^{18}O_{sa/VSMOW}$  and  $\delta^{17}O_{sa/VSMOW}$  from measurements of the  $^{14}N^{18}O^{16}O_2$  and  $^{14}N^{17}O^{16}O_2$  peaks relative to the  $^{14}N^{16}O_3$  peak (“M0”).

To benchmark  $\delta$ -values for isotopologs measured in the “without M0” experiment, we leverage information known independently from gas-source IRMS measurements. For instance,  $\delta^{18}O_{sa/N11}$  was calculated from  $^{15}N^{16}O_3$  and  $^{14}N^{18}O^{16}O_2$  isotopologs measured “without M0”, using  $\delta^{15}N_{sa/N11}$  known from IRMS (note, however, that  $\delta^{15}N_{sa/N11}$  can also be determined by ESI; Eq. 1):



$$\delta(^{18}\text{O}/^{15}\text{N})_{sa/N11} = \frac{(^{14}\text{N}^{18}\text{O}^{16}\text{O}_2/^{15}\text{N}^{16}\text{O}_3)_{sample}}{(^{14}\text{N}^{18}\text{O}^{16}\text{O}_2/^{15}\text{N}^{16}\text{O}_3)_{N11}} - 1 \quad (4)$$

$$\delta^{18}\text{O}_{sa/N11} = \delta(^{18}\text{O}/^{15}\text{N})_{sa/N11} + \delta^{15}\text{N}_{sa/N11} + \delta(^{18}\text{O}/^{15}\text{N})_{sa/N11} \cdot \delta^{15}\text{N}_{sa/N11} \quad (5)$$

The “without M0” experiment is conceptually novel and requires appropriate calibration procedures. 1- or 2-point calibrations were applied to the  $\delta$ -scale that was directly measured, for example for  $\delta(^{18}\text{O}/^{15}\text{N})$  using USGS32 and N11. These two materials have a similar  $\delta^{18}\text{O}$  but are suitable for calibration here because they display a big difference in  $^{14}\text{N}^{18}\text{O}^{16}\text{O}_2/^{15}\text{N}^{16}\text{O}_3$  due to their difference in  $\delta^{15}\text{N}$ . A more detailed description of  $\delta$ -value calculations, including a formal derivation of equations and a sensitivity analysis of deviations associated with approximating  $\delta$ -values from isotopolog intensities is available in the SI.

## Results and Discussion

### Observable isotopic species

There are twenty stable isotopic species of nitrate ( $\text{NO}_3^-$ ) with distinct masses (Table 1). The monoisotopic peak (M0; representing nitrate containing only the light isotopes  $^{14}\text{N}$  and  $^{16}\text{O}$ ) as well as three peaks that contain ions with a single substitution of  $^{15}\text{N}$ ,  $^{17}\text{O}$  or  $^{18}\text{O}$  are readily detected by ESI-Orbitrap (Figure 1). M0 contains almost all of the nitrate ions (98.9 %). Other rare isotopologs are within the noise and not detected in these spectra, because the Orbitrap has a limited capacity for ions per scan.

To study rare isotopologs, the monoisotopic peak can be eliminated by adjusting the quadrupole mass filter,<sup>11</sup> a setting which we call the “without M0” experiment. This enrichment allows the detection of the doubly-substituted species  $^{15}\text{N}^{18}\text{O}^{16}\text{O}_2$ ,  $^{14}\text{N}^{17}\text{O}^{18}\text{O}^{16}\text{O}$  and  $^{14}\text{N}^{18}\text{O}_2^{16}\text{O}$ . Analysis of such “clumped” isotopes has gained prominence in geochemistry, for example in carbonate

clumped isotope thermometry.<sup>30</sup> Nitrate clumped isotopes, however, have never been measured before. This highlights that quantifying intact polar solutes can open new avenues for research.

## Relations to conventional isotope scales

The measurements described here differ in important aspects from two approaches that are common in various scientific disciplines. Intact polar molecules such as metabolites are often studied using artificially enriched isotope tracers. The abundances of isotopically labeled compounds are quantified by mass isotopomer analysis or related metabolic flux analyses.<sup>31,32</sup> In contrast, natural isotopic variations occur without experimental interference and are orders of magnitude smaller. Natural isotopic differences are thus usually measured after converting a polar analyte into a gas to achieve the necessary precision. Relative stable isotope abundances are then quantified as ratios in comparison to those of an internationally accepted reference, expressed in parts per thousand (permil; ‰). For example, the nitrogen-15 content of nitrate in a sample normally is determined by converting the nitrate to another compound (e.g., N<sub>2</sub>, N<sub>2</sub>O), and the IRMS results are reported as a  $\delta^{15}\text{N}$  value, using nitrogen in air as the primary reference ( $\delta^{15}\text{N}$  of air N<sub>2</sub>  $\equiv$  0):

$$\delta^{15}\text{N} = \frac{(^{15}\text{N}/^{14}\text{N})_{\text{sample}} - (^{15}\text{N}/^{14}\text{N})_{\text{AIR}}}{(^{15}\text{N}/^{14}\text{N})_{\text{AIR}}} = \frac{(^{15}\text{N}/^{14}\text{N})_{\text{sample}}}{(^{15}\text{N}/^{14}\text{N})_{\text{AIR}}} - 1 \quad (6)$$

When we are now measuring intact nitrate, each N and O isotope can be found in multiple isotopologs. In the simplest case, <sup>15</sup>N occurs in 10 species that have distinct masses (Table 1). This combinatorial complexity is a fundamental property of compounds containing elements with more than one atomic mass and it includes isotopic fingerprints that are largely unexplored.<sup>33</sup> Any deviations from the stochastic isotopolog pattern are exceedingly small for the most abundant isotopologs of unlabeled nitrate. It can thus be approximated that:

$$\frac{^{15}\text{N}}{^{14}\text{N}} = \frac{^{15}\text{N}^{16}\text{O}_3}{^{14}\text{N}^{16}\text{O}_3} \quad (7)$$

This relation can be used to define nitrate  $\delta^{15}\text{N}$  based on measuring nitrate isotopologs from a sample and a reference. Because atmospheric  $\text{N}_2$  cannot be measured directly by ESI, an additional calculation step is needed to express  $\delta^{15}\text{N}$  values in the established international reference frame versus air  $\text{N}_2$  (see Materials and Methods and SI).

The “without M0” experiment enriches rare isotopologs about 100-fold in the Orbitrap, with corresponding increases in counting statistics (shot noise limit). For example,  $^{14}\text{N}^{18}\text{O}^{16}\text{O}_2/^{15}\text{N}^{16}\text{O}_3$  can be constrained with a precision of  $\pm 0.1\%$  (1 RSE) within 15 min. Such tightly determined ratios offer much potential for isotope analysis. For instance, unusual isotopolog ratios can be leveraged to obtain familiar isotope ratios, using a relation of this kind:

$$\frac{^{18}\text{O}}{^{16}\text{O}} = \frac{^{14}\text{N}^{18}\text{O}^{16}\text{O}_2}{^{15}\text{N}^{16}\text{O}_3} \cdot \frac{^{15}\text{N}}{^{14}\text{N}} \quad (8)$$

The link to conventional bulk isotopic ratios here is established by adding a single independent parameter for the bulk isotopic composition. This can be a  $\delta^{18}\text{O}$  or  $\delta^{15}\text{N}$  value determined in the “with M0” experiment, thus using ESI as a stand-alone method. For benchmarking “without M0” experiments, we can alternatively utilize  $\delta$ -values independently known from gas-source IRMS analysis (see also Materials and Methods and SI).

### Necessary requirements for IRMS fidelity

Isotope ratios need to be measured with high accuracy and precision to learn about natural systems. Appropriate measurement principles have been refined for IRMS over decades. In the following we bring these principles to the ESI-Orbitrap and report several features that are critical for its use as an isotope ratio mass spectrometer.

The measurement was automated, which improves reproducibility and sample throughput, and decreases the sample amount needed per measurement. Most important for isotope ratios, automation should keep electrospray conditions as stable as possible during data collection. We configured a HPLC system to sequentially deliver samples by “flow injection” via a continuous flow to the electrospray needle (Figure 2). After stabilization for 1-2 hours, this setup allows about 100 sample injections for isotope analysis per day. One injection requires less than 50  $\mu\text{L}$  of a 50  $\mu\text{M}$  sample ( $<155$  ng of  $\text{NO}_3^-$ ), with room for further improvements. This sample throughput and sensitivity is comparable to well-established methods for nitrate isotope analysis, like the denitrifier method.<sup>19</sup>

This automation enables systematic tests to improve the fidelity of ESI-Orbitrap as an IRMS. For instance, long series of replicate analyses showed that in some datasets isotope ratios were impacted by a small but significant system drift over time, which can be characterized and corrected using multiple blocks of reference injections (Figure 2D; Materials and Methods, SI). A memory test with differences in  $\delta$ -values of up to 149 ‰ between sample blocks showed no significant memory effects with an average of  $0.02 \text{ ‰} \pm 0.5 \text{ ‰}$  (Figure 2E and SI). Experiments also indicate that  $\delta$ -values are constant over a range of nitrate concentrations. For example,  $\delta^{15}\text{N}$  is constant for samples between 25 and 200  $\mu\text{M}$  in comparisons with a reference at 50  $\mu\text{M}$  (Figure 2C). For lower sample concentrations, the reference should be diluted as well. This is in line with the “principle of identical treatment”, which conveys that isotope measurements become more accurate with lesser differences between sample and reference.

## Validation with reference materials

To assess precision and accuracy of this new setup, three USGS nitrate reference materials were measured against our lab standard N11 (Table 2). Sequences with 4 sample replicates at 3 different days revealed an average precision in the “with M0” experiments of 0.23 ‰ for  $\delta^{15}\text{N}_{\text{AIR}}$ , 0.4 ‰ for  $\delta^{18}\text{O}_{\text{VSMOW}}$ , and 0.3 ‰ for  $\delta^{17}\text{O}_{\text{VSMOW}}$ . The “without M0” experiment yielded  $\delta^{17}\text{O}_{\text{VSMOW}}$  and

$\delta^{18}\text{O}_{\text{VSMOW}}$  values with  $< 0.4 \text{ ‰}$  precision, respectively (using  $^{15}\text{N}^{16}\text{O}_3$  as denominator); these values include error propagation of a  $\delta^{15}\text{N}_{\text{sa/N11}}$  precision of  $\pm 0.23 \text{ ‰}$  to fully reflect uncertainties associated with using ESI as a stand-alone technique, as  $\delta^{15}\text{N}$  from IRMS were used here to calculate  $\delta^{17}\text{O}_{\text{VSMOW}}$  and  $\delta^{18}\text{O}_{\text{VSMOW}}$  to benchmark the procedure. Overall, this precision is promising and within the requirements of many research applications.

This performance well below  $1 \text{ ‰}$  isotopic precision for nitrate  $\delta^{15}\text{N}_{\text{AIR}}$ ,  $\delta^{17}\text{O}_{\text{VSMOW}}$  and  $\delta^{18}\text{O}_{\text{VSMOW}}$  provides a first starting point for developing dedicated referencing schemes and procedures that are optimized for accurate nitrate isotopolog analysis. For example, we can account for a subtle but reproducible contraction of the  $\delta^{15}\text{N}$  scale, relative to the values established by conventional IRMS. The scale contraction on  $\delta^{15}\text{N}$  was approximately  $1.2 \text{ ‰}$  per  $100 \text{ ‰}$ . This was observed on several instruments across multiple laboratories.<sup>12</sup> Correcting this scale contraction by 2-point calibration improved the accuracy of the reported  $\delta^{15}\text{N}_{\text{AIR}}$  (see Materials and Methods, SI). Scale corrections were also applied for the other  $\delta$ -values. Measured  $\delta^{18}\text{O}$  values determined in this study were most consistent with independent data for reference materials (Table 2) and other samples (see below) if the lab standard N11 was assigned a  $\delta^{18}\text{O}$  value of  $+26.7 \text{ ‰}$ , which is slightly higher than the expected value of  $+26.3 \text{ ‰}$  ( $\delta^{17}\text{O}$  was not reassigned; see also Table S2). Measured  $\delta^{18}\text{O}$  values determined "with M0" required an additional offset of  $0.36 \text{ ‰}$  to match independent data more consistently. Causes of these minor offsets could include uncertainties in the reference data<sup>22,34</sup> and artifacts of the ESI-Orbitrap system (see also section about current limitations). With isotopic referencing schemes and calibrations, most measured values were consistent with the expected values within  $\pm 1 \text{ SD}$  (Table 2).<sup>34</sup> These benchmarking efforts highlight that with further technical refinements ESI-Orbitrap can become a new generation of highly accurate IRMS technology.

Nitrate that forms via photochemical reactions in the atmosphere carries a characteristic anomaly, 'mass-independent' fractionation of  $^{16}\text{O}/^{17}\text{O}/^{18}\text{O}$ .<sup>35</sup> The anomaly commonly is expressed as  $\Delta^{17}\text{O}$ ,

the excess  $^{17}\text{O}$  over what is normally expected based on  $^{18}\text{O}$  content ( $\Delta^{17}\text{O} \cong \delta^{17}\text{O} - 0.52 \times \delta^{18}\text{O}$ ). The precision of  $\Delta^{17}\text{O}$  is limited by the precision of the  $\delta^{17}\text{O}$  and  $\delta^{18}\text{O}$ , which can be constrained by ESI most efficiently in the “without M0” experiment, in combination with independently known  $\delta^{15}\text{N}$  values. The obtained  $\Delta^{17}\text{O}$  values agree to within  $\pm 0.4\text{‰}$  with the values from gas-source IRMS (Table 2). It is important to note, however, that previous calibrations and definitions of  $\Delta^{17}\text{O}$  in nitrate may not be entirely consistent, which may affect accuracy comparisons. While existing methods for  $\Delta^{17}\text{O}$  measurements by gas-source isotope ratio mass spectrometry can be more precise, ESI-Orbitrap makes this measurement readily accessible and enables exploration of  $\Delta^{17}\text{O}$  variations that range from near 0 ‰ in biogenic nitrate to more than 30 ‰ in atmospheric nitrate. Additionally,  $^{17}\text{O}$  anomalies could also be detected from doubly-substituted, oxygen-containing isotopologs such as  $^{14}\text{N}^{17}\text{O}^{18}\text{O}^{16}\text{O}$  and  $^{14}\text{N}^{18}\text{O}_2^{16}\text{O}$ , which are only accessible by ESI-Orbitrap. Together these tests with nitrate reference materials suggest that ESI-Orbitrap enables comprehensive and high-accuracy isotopic analysis of nitrate and likely other oxyanions such as sulfate.<sup>12</sup>

## Current limitations of technology development

The proposed ESI technology aims to build a technology bridge that will make it possible to use a sophisticated bioanalytical technique (ESI-Orbitrap MS) for powerful geochemical concepts (isotopic fingerprints in molecules). Progress on this effort is necessarily incremental, as all components of sample preparation, analysis and data interpretation need to be adapted or reinvented. Some limitations we now encounter are likely linked to data acquisition and signal processing. For example, the stability of electrospray can vary between days and sometimes even samples, and needs to be better controlled. Also, more technical development is needed to better control the quantification of isotopolog signals. The automatic processing procedure of the Orbitrap signal has been developed for untargeted mass spectrometry, which has very different performance requirements than isotope ratio analysis.<sup>36</sup> This may explain variabilities we notice

in certain signals that seem prone to artefacts at the sub-permil isotopic accuracy level, most notably  $^{14}\text{N}^{18}\text{O}^{16}\text{O}_2$  in the “with M0” experiment.  $\delta^{18}\text{O}$  values in the “with M0” experiment were consistently shifted on average by  $-0.36\text{‰}$  relative to the “without M0” experiment (Table 2 and SI). This unexplained impact on the quantification of the  $^{14}\text{N}^{18}\text{O}^{16}\text{O}_2$  signal in the “with M0” experiment motivates an ongoing detailed investigation on the Orbitrap signal processing.

These limitations are unlikely to remain at the level experienced today as it may still be possible to make systematic progress by adapting underlying procedures or instrument components. The major next question for future research applications is rather whether similar accuracy can be obtained from environmental samples and whether new biogeochemical information will become measurable. The following is a first rigorous attempt at these questions, using vetted samples for benchmarking.

## Validation with environmental samples

Nitrate occurs in many diverse environments on Earth. The use of ESI-Orbitrap as IRMS hence hinges on its ability to measure isotopes reliably in the face of influences of the sample other than the analyte that is being measured (i.e., matrix effects). There are well established protocols to isolate nitrate as silver salt from diverse sample types, including groundwater and brines.<sup>21,26,37,38</sup> To gain insights into how the ESI method can be used for environmental samples in the absence of interfering anions, we measured several samples with known and highly variable  $\delta^{15}\text{N}$ ,  $\delta^{18}\text{O}$  and  $\delta^{17}\text{O}$  values that were prepared as  $\text{AgNO}_3$ .

A selection of 3  $\text{AgNO}_3$  samples was analyzed by flow injection ( $n = 4$ ) against N11 in the  $\text{AgNO}_3$  form. This sequence was repeated on 3 different days to evaluate the long-term reproducibility. The reproducibility in the “with M0” experiments was  $<0.3\text{‰}$  for  $\delta^{15}\text{N}_{\text{AIR}}$ . In the “without M0” experiments,  $\delta^{17}\text{O}_{\text{VSMOW}}$  and  $\delta^{18}\text{O}_{\text{VSMOW}}$  were also determined with reproducibility of  $<0.5\text{‰}$  and  $<0.3\text{‰}$  with  $^{15}\text{N}^{16}\text{O}_3$  as denominator (Table 3). Most results fall within  $\pm 1$  SD of the expected

values after being calibrated using  $\text{AgNO}_3$  reference values and  $\delta$  scaling factors from Table S1. These tests illustrate that the ESI-Orbitrap can be used for isotope analysis of analytes from complex geological or biological samples in combination with suitable extraction methods.

ESI-Orbitrap could be useful for high-throughput studies. To characterize its performance in a screening mode, 7  $\text{AgNO}_3$  samples representing natural nitrate with a wide range of isotopic compositions, including variable  $\Delta^{17}\text{O}$ , were analyzed by a single injection. The sample Antarctica (N17449) was injected several times during the sequence and used to determine  $\delta$  scaling factors for 2-point calibration. Each block of four samples was bracketed by two blocks of reference injections (N11 in the  $\text{AgNO}_3$  form). The average precision of the reference injections was  $\pm 0.9\text{‰}$  (1 SD) for  $\delta^{15}\text{N}_{\text{AIR}}$  in the “with M0” experiment and also for  $\delta^{18}\text{O}_{\text{VSMOW}}$  in the “without M0” experiment; the precision for  $\delta^{17}\text{O}_{\text{VSMOW}}$  in the “without M0” experiment was  $\pm 0.4\text{‰}$  (1 SD). Most data fall within  $\pm 2$  SD to the expected values (Table S1). The performance for oxygen isotopes in these tests was more robust in the “without M0” experiment. Reported  $\delta^{18}\text{O}$  and  $\delta^{17}\text{O}$  were thus calculated using the  $^{14}\text{N}^{18}\text{O}^{16}\text{O}_2/^{15}\text{N}^{16}\text{O}_3$  and  $^{14}\text{N}^{17}\text{O}^{16}\text{O}_2/^{15}\text{N}^{16}\text{O}_3$  ratios together with independently known  $\delta^{15}\text{N}$  values (Table S2). These tests of environmental samples provide a proof-of-concept demonstration that ESI-Orbitrap can yield reliable results for environmental samples, adding crucial evidence that it can become a broadly useful IRMS technology.

### ‘Dilute and shoot’

Results summarized above were obtained from various types of environmental samples that had been prepared in the form of purified  $\text{AgNO}_3$ . This purification is time consuming and can itself introduce uncertainties (e.g., blanks, fractionations) that are comparable to analytical uncertainties in the method validation discussed above. Much more practical would be a method that does not require pre-purification for routine isotopic analyses of  $\text{NO}_3^-$ . This is not a trivial task, as there can be adverse effects of the sample matrix on isotope ratio measurements.<sup>39</sup> In addition, salts can be



deposited during ESI, so that the spray needle tip and the capillary transfer tube need to be exchanged, the mass spectrometer vented, and the ion optics cleaned.<sup>40</sup>

Electrospray ionization is able to detect minute quantities of analytes, down into the low-attomolar range ( $10^{-18}$  mol).<sup>41</sup> It is therefore possible to dilute nitrate samples substantially for isotopic analysis. In some environmental sample types dilution may allow to overcome the negative impacts of interfering ions, because dilution decreases the competition of analyte and matrix for ionization.<sup>42</sup> To this end, we conducted preliminary tests with some of the brines, soil leachate solutions, and representative groundwater samples with different relative concentrations of  $\text{NO}_3^-$  and other ions (Table 4).

Groundwaters, leachates and brines were diluted in pure methanol to 1  $\mu\text{M}$  (62  $\text{pg}/\mu\text{L}$  of nitrate). We analyzed the diluted samples by direct infusion via a setup analogous to a ‘dual inlet’ system used in gas-source IRMS (Figure 3A). This “dilute and shoot” method is a safe and simple way to study new unpurified environmental sample types that could damage or contaminate the flow injection system. Nitrate concentrations  $<1$   $\mu\text{M}$  were not desirable in our setup, because nitrate blanks from reagents and waters typically were on the order of 0.2  $\mu\text{M}$  under our lab conditions. Tests with USGS reference materials revealed a blank contribution of ca. 18 % for a 1  $\mu\text{M}$  sample. Based on this estimate we calculated empirical  $\delta^{18}\text{O}$  and  $\delta^{17}\text{O}$  values of 36 ‰ and 13 ‰ for the blank in the environmental sample set by applying a best fit between apparent and expected  $\delta$ -values (no constraint for  $\Delta^{17}\text{O}$  was applied). This information was used to correct for background contributions at low nitrate concentrations. Because blank contributions are an important aspect of many research questions, alternative correction procedures should be evaluated in future studies.

Environmental samples with concentrations  $>500$   $\mu\text{M}$  nitrate and with up to a 100-fold excess of chloride over nitrate were analyzed using alternating 10 min. infusions (40 pmol nitrate each) of sample and reference (Figure 3). The average precision based on the agreement with expected

values for  $\delta^{15}\text{N}_{\text{AIR}}$  was 1.1 ‰ and for  $\delta^{18}\text{O}_{\text{VSMOW}}$  was 0.7 ‰ (Table 4). A potential contaminant for the  $^{14}\text{N}^{17}\text{O}^{16}\text{O}_2$  signal at a resolution of  $R = 15,000$  is the  $^{18}\text{O}$ -isotopolog of bicarbonate. With a higher resolution ( $R=30,000$ ) the  $\delta^{17}\text{O}_{\text{VSMOW}}$  was determined with an average precision of 2.2 ‰. The  $^{14}\text{N}^{17}\text{O}^{16}\text{O}_2$  isotopolog requires further investigation of interfering masses and signal processing.

In sum, these tests with environmental nitrate illustrate that ESI-Orbitrap could become a very sensitive and useful tool to obtain initial overviews when many samples need to be analyzed. ‘Dilute and shoot’ could be especially promising for studies of certain sample types, like nitrate in terrestrial ecosystems. Additionally, the dual inlet is a cost-effective way of turning a large number of existing ESI-Orbitrap instruments into isotope ratio mass spectrometers, providing a mechanism to kick-start and democratize new technology and method developments that can advance isotope analytics.

### New dimensions: clumped isotopes of nitrate

The study of isotopes in intact molecules by ESI permits numerous opportunities to discover new isotopic signatures in polar organic and inorganic solutes. For example, non-statistical isotopic distributions among nitrate molecules (i.e., “clumped isotopes”) may encode how individual pathways of the biogeochemical N cycling interact and shape the nitrogen cycle.<sup>43</sup> To first order, the three clumped isotope signals in the “without M0” experiment offer multiple paths to approximate  $^{15}\text{N}$ ,  $^{18}\text{O}$  and  $^{17}\text{O}$  contents. Thus, the “without M0” experiment yields *apparent*  $\delta$ -values that can be skewed by isotope clumping and other processes. For example,

$$\frac{^{15}\text{N}}{^{14}\text{N}} \approx \frac{^{15}\text{N}^{18}\text{O}^{16}\text{O}_2}{^{14}\text{N}^{18}\text{O}^{16}\text{O}_2} \quad (9)$$

Measuring the nitrate reference materials in the “without M0” experiment confirms that such ratios indeed yield  $\delta$ -values that are close to the true  $\delta^{15}\text{N}$  and  $\delta^{18}\text{O}$  values (Table 5). However, it is possible that some of the apparent  $\delta$ -values are skewed by processes that cause non-stochastically

distributed isotopolog populations. For USGS32, for example, it appears that  $\delta(^{15}\text{N}^{18}\text{O}/^{18}\text{O})$  is 5.6 ‰ lower than the actual  $\delta^{15}\text{N}$  and  $\delta(^{15}\text{N}^{18}\text{O}/^{15}\text{N})$  is 1.6 ‰ lower than the actual  $\delta^{18}\text{O}$ . In contrast, the apparent  $\delta(^{17}\text{O}^{18}\text{O}/^{18}\text{O})$  value of the Antarctica leachate analyzed as  $\text{AgNO}_3$  salt is +8.7 ‰ higher than the actual  $\delta^{17}\text{O}$  value. Whether these are indeed anomalies containing useful information about the formation processes of nitrate requires further studies. Nonetheless, our preliminary analyses of isotope clumping in nitrates suggest that new isotopic dimensions of nitrate can be measured with a precision that may be suitable to evaluate whether significant nitrate clumping effects manifest in nature.

## Conclusions

This study illustrates how the ESI-Orbitrap can be repurposed as an isotopic ratio mass spectrometer. ESI-Orbitrap is capable of differentiating nitrates of different origins, such as from atmospheric, anthropogenic and biogenic processes. Results indicate the ESI-Orbitrap method can provide usable data for conventional isotope ratios in nitrate ( $\delta^{15}\text{N}$ ,  $\delta^{18}\text{O}$ ,  $\Delta^{17}\text{O}$ ), potentially on much smaller samples and with less sample preparation than previous methods. Measurements of seven nitrate isotopologs provide multiple pathways for calculating the conventional  $\delta$ -values including non-mass-dependent oxygen isotopic variation, as well as opportunities to explore non-random isotopic distributions (i.e., clumping) in nitrate. In summary, this report provides initial theoretical frameworks, measurement principles, and validation procedures for high-fidelity isotope measurements by ESI-Orbitrap on nitrate, with potential applications to diverse intact polar solutes.

## Supporting Information

The Supporting Information (SI) is available free of charge. It contains (1) a description of data sets and data processing, (2) supplemental tables, (3) derivation of equations for calculating  $\delta$ -values from ratios of isotopologs and sensitivity analysis associated with approximating  $\delta$ -values

from isotopolog intensities, (4) a spreadsheet with details on  $\delta$  scale contraction/expansion, memory effects, linearity, and drift, and (5) a spreadsheet with details on reproducibility, precision, accuracy of isotope measurements in nitrate reference materials.

## Acknowledgments

The authors thank Neil Sturchio and two anonymous reviewers for helpful comments on the manuscript. We thank Alexander Makarov, Uwe Rickens, Dieter Juchelka, John Eiler, and Nathan Dalleska for fruitful discussions. A. H. likes to thank Tabiwang Arrey, Colin Wirth, Christian Klaas, Knut Spengler and Charles Cartwright for their help in the lab. This study was made possible by a grant from the National Science Foundation (Award #2041539 to S.K. and C.N.) and a fellowship by the Hanse-Wissenschaftskolleg (HWK) Institute for Advanced Study (C.N.). Participation of J.K.B. and S.J.M. was supported in part by the U.S. Geological Survey Water Mission Area and Environmental Health Mission Area. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

## Notes

The authors declare the following competing financial interests: A.H., K.L.F. and K.A. are employees of Thermo Fisher Scientific, which manufactures Orbitrap mass spectrometers as well as gas-source isotope ratio mass spectrometers. Published data will also be made available through the BCO-DMO data repository (<https://www.bco-dmo.org>; project 851883).

## Author Contributions

A.H. and C.N. initiated the project and carried out the ESI-Orbitrap experiments. J.K.B. and S.J.M. supplied samples and closely participated in data analysis. K.A. developed key analysis software, K.L.F. assisted in configuring the research prototype mass spectrometer in Bremen. X.T.W.

suggested the project and provided advice on experiments, S.H.K. established theory for  $\delta$ -value calculations. All authors participated in the interpretation of experimental data and writing of the manuscript.

## References

- (1) Brand, W. A.; Douthitt, C. B.; Fourel, F.; Maia, R.; Rodrigues, C.; Maguas, C.; Prohaska, T. Gas Source Isotope Ratio Mass Spectrometry (IRMS). In *Sector Field Mass Spectrometry for Elemental and Isotopic Analysis*; Prohaska, T., Irrgeher, J., Zitek, A., Jakubowski, N., Eds.; 2015; pp 500–549.
- (2) Crosson, E. R.; Ricci, K. N.; Richman, B. A.; Chilese, F. C.; Owano, T. G.; Provencal, R. A.; Todd, M. W.; Glasser, J.; Kachanov, A. A.; Paldus, B. A.; Spence, T. G.; Zare, R. N. Stable Isotope Ratios Using Cavity Ring-down Spectroscopy: Determination of  $^{13}\text{C}/^{12}\text{C}$  for Carbon Dioxide in Human Breath. *Anal. Chem.* **2002**, *74* (9), 2003–2007.
- (3) Guyader, S.; Thomas, F.; Jamin, E.; Grand, M.; Akoka, S.; Silvestre, V.; Remaud, G. S. Combination of  $^{13}\text{C}$  and  $^2\text{H}$  SNIF-NMR Isotopic Fingerprints of Vanillin to Control Its Precursors. *Flavour Fragr. J.* **2019**, *34* (2), 133–144.
- (4) Abelson, P. H.; Hoering, T. C. Carbon Isotope Fractionation in Formation of Amino Acids by Photosynthetic Organisms. *Proc. Natl. Acad. Sci. U. S. A.* **1961**, *47*, 623–632.
- (5) Knolhoff, A. M.; Callahan, J. H.; Croley, T. R. Mass Accuracy and Isotopic Abundance Measurements for HR-MS Instrumentation: Capabilities for Non-Targeted Analyses. *J. Am. Soc. Mass Spectrom.* **2014**, *25* (7), 1285–1294.
- (6) Fischer, C. R.; Bowen, B. P.; Pan, C.; Northen, T. R.; Banfield, J. F. Stable-Isotope Probing Reveals That Hydrogen Isotope Fractionation in Proteins and Lipids in a Microbial Community Are Different and Species-Specific. *ACS Chem. Biol.* **2013**, *8* (8), 1755–1763.
- (7) Neubauer, C.; Sessions, A. L.; Booth, I. R.; Bowen, B. P.; Kopf, S. H.; Newman, D. K.; Dalleska, N. F. Towards Measuring Growth Rates of Pathogens during Infections by  $\text{D}_2\text{O}$ -Labeling Lipidomics. *Rapid Commun. Mass Spectrom.* **2018**, *32* (24), 2129–2140.
- (8) Herath, K. B.; Zhong, W.; Yang, J.; Mahsut, A.; Rohm, R. J.; Shah, V.; Castro-Perez, J.; Zhou, H.; Attygalle, A. B.; Kang, L.; Others. Determination of Low Levels of  $^2\text{H}$ -Labeling Using High-Resolution Mass Spectrometry: Application in Studies of Lipid Flux and beyond. *Rapid Commun. Mass Spectrom.* **2014**, *28* (3), 239–244.
- (9) Castro-Perez, J.; Previs, S. F.; McLaren, D. G.; Shah, V.; Herath, K.; Bhat, G.; Johns, D. G.; Wang, S.-P.; Mitnaul, L.; Jensen, K.; Vreeken, R.; Hankemeier, T.; Roddy, T. P.; Hubbard, B. K. In Vivo  $\text{D}_2\text{O}$  Labeling to Quantify Static and Dynamic Changes in Cholesterol and Cholesterol Esters by High Resolution LC/MS. *J. Lipid Res.* **2011**, *52* (1), 159–169.
- (10) Eiler, J.; Cesar, J.; Chimiak, L.; Dallas, B.; Grice, K.; Griep-Raming, J.; Juchelka, D.; Kitchen, N.; Lloyd, M.; Makarov, A.; Robins, R.; Schwieters, J. Analysis of Molecular Isotopic Structures at High Precision and Accuracy by Orbitrap Mass Spectrometry. *Int. J. Mass Spectrom.* **2017**, *422*, 126–142.
- (11) Neubauer, C.; Sweredoski, M. J.; Moradian, A.; Newman, D. K.; Robins, R. J.; Eiler, J. M. Scanning the Isotopic Structure of Molecules by Tandem Mass Spectrometry. *Int. J. Mass Spectrom.* **2018**, *434*, 276–286.
- (12) Neubauer, C.; Crémière, A.; Wang, X. T.; Thiagarajan, N.; Sessions, A. L.; Adkins, J. F.; Dalleska, N. F.; Turchyn, A. V.; Clegg, J. A.; Moradian, A.; Others. Stable Isotope Analysis of Intact Oxyanions Using Electrospray Quadrupole-Orbitrap Mass Spectrometry. *Anal. Chem.* **2020**, *92* (4), 3077–3085.
- (13) Makarov, A.; Grinfeld, D.; Ayzikov, K. Fundamentals of Orbitrap Analyzer. In *Fundamentals and Applications of Fourier Transform Mass Spectrometry*; Kanawati, B., Schmitt-Kopplin, P., Eds.; Elsevier, 2019; pp 37–61.
- (14) Fowler, D.; Coyle, M.; Skiba, U.; Sutton, M. A.; Cape, J. N.; Reis, S.; Sheppard, L. J.; Jenkins, A.; Grizzetti, B.; Galloway, J. N.; Vitousek, P.; Leach, A.; Bouwman, A. F.; Butterbach-Bahl, K.; Dentener, F.; Stevenson, D.; Amann, M.; Voss, M. The Global Nitrogen Cycle in the Twenty-First

- Century. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **2013**, 368 (1621), 20130164.
- (15) Zhang, X.; Ward, B. B.; Sigman, D. M. Global Nitrogen Cycle: Critical Enzymes, Organisms, and Processes for Nitrogen Budgets and Dynamics. *Chem. Rev.* **2020**, 120 (12), 5308–5351.
- (16) Sigman, D. M.; Casciotti, K. L.; Andreani, M.; Barford, C.; Galanter, M.; Böhlke, J. K. A Bacterial Method for the Nitrogen Isotopic Analysis of Nitrate in Seawater and Freshwater. *Anal. Chem.* **2001**, 73 (17), 4145–4153.
- (17) Casciotti, K. L.; Sigman, D. M.; Hastings, M. G.; Böhlke, J. K.; 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.
- (18) Coplen, T. B.; Böhlke, J. K.; Casciotti, K. L. Using Dual-Bacterial Denitrification to Improve  $\delta^{15}\text{N}$  Determinations of Nitrates Containing Mass-Independent  $^{17}\text{O}$ . *Rapid Commun. Mass Spectrom.* **2004**, 18 (3), 245–250.
- (19) Weigand, M. A.; Foriel, J.; Barnett, B.; Oleynik, S.; Sigman, D. M. Updates to Instrumentation and Protocols for Isotopic Analysis of Nitrate by the Denitrifier Method. *Rapid Commun. Mass Spectrom.* **2016**, 30 (12), 1365–1383.
- (20) Böhlke, J. K.; Coplen, T. B. *Interlaboratory Comparison of Reference Materials for Nitrogen-Isotope-Ratio Measurements*; IAEA-TECDOC-825; IAEA, 1995.
- (21) Michalski, G.; Savarino, J.; Böhlke, J. K.; Thiemens, M. Determination of the Total Oxygen Isotopic Composition of Nitrate and the Calibration of a  $\Delta^{17}\text{O}$  Nitrate Reference Material. *Anal. Chem.* **2002**, 74 (19), 4989–4993.
- (22) Böhlke, J. K.; Mroczkowski, S. J.; Coplen, T. B. Oxygen Isotopes in Nitrate: New Reference Materials for  $^{18}\text{O}$ :  $^{17}\text{O}$ :  $^{16}\text{O}$  Measurements and Observations on Nitrate-Water Equilibration. *Rapid Commun. Mass Spectrom.* **2003**, 17 (16), 1835–1846.
- (23) Sigman, D. M.; Fripiat, F. Nitrogen Isotopes in the Ocean. *Encyclopedia of Ocean Sciences*. 2019, pp 263–278.
- (24) Casciotti, K. L. Nitrogen and Oxygen Isotopic Studies of the Marine Nitrogen Cycle. *Ann. Rev. Mar. Sci.* **2016**, 8, 379–407.
- (25) Jackson, W. A.; Böhlke, J. K.; Andraski, B. J.; Fahlquist, L.; Bexfield, L.; Eckardt, F. D.; Gates, J. B.; Davila, A. F.; McKay, C. P.; Rao, B.; Sevanthi, R.; Rajagopalan, S.; Estrada, N.; Sturchio, N.; Hatzinger, P. B.; Anderson, T. A.; Orris, G.; Betancourt, J.; Stonestrom, D.; Latorre, C.; Li, Y.; Harvey, G. J. Global Patterns and Environmental Controls of Perchlorate and Nitrate Co-Occurrence in Arid and Semi-Arid Environments. *Geochim. Cosmochim. Acta* **2015**, 164, 502–522.
- (26) Jackson, W. A.; Böhlke, J. K.; Gu, B.; Hatzinger, P. B.; Sturchio, N. C. Isotopic Composition and Origin of Indigenous Natural Perchlorate and Co-Occurring Nitrate in the Southwestern United States. *Environ. Sci. Technol.* **2010**, 44 (13), 4869–4876.
- (27) Böhlke, J. K.; Hatzinger, P. B.; Sturchio, N. C.; Gu, B.; Abbene, I.; Mroczkowski, S. J. Atacama Perchlorate as an Agricultural Contaminant in Groundwater: Isotopic and Chronologic Evidence from Long Island, New York. *Environ. Sci. Technol.* **2009**, 43 (15), 5619–5625.
- (28) Böhlke, J. K.; Denver, J. M. 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.
- (29) Hannon, J. E.; Böhlke, J. K.; Mroczkowski, S. J. Effects of Nitrate and Water on the Oxygen Isotopic Analysis of Barium Sulfate Precipitated from Water Samples. *Rapid Commun. Mass Spectrom.* **2008**, 22 (24), 4109–4120.
- (30) Eiler, J. M. Paleoclimate Reconstruction Using Carbonate Clumped Isotope Thermometry. *Quat. Sci. Rev.* **2011**, 30 (25), 3575–3588.
- (31) Hellerstein, M. K.; Neese, R. A. Mass Isotopomer Distribution Analysis at Eight Years: Theoretical, Analytic, and Experimental Considerations. *Am. J. Physiol.* **1999**, 276 (6 Pt 1), E1146–E1170.
- (32) Jang, C.; Chen, L.; Rabinowitz, J. D. Metabolomics and Isotope Tracing. *Cell* **2018**, 173 (4), 822–837.
- (33) Eiler, J. M. The Isotopic Anatomies of Molecules and Minerals. *Annu. Rev. Earth Planet. Sci.* **2013**, 41 (1), 411–441.
- (34) Brand, W. A.; Coplen, T. B.; Aerts-Bijma, A. T.; Böhlke, J. K.; Gehre, M.; Geilmann, H.; Gröning, M.; Jansen, H. G.; Meijer, H. A. J.; Mroczkowski, S. J.; Others. Comprehensive Inter-Laboratory Calibration of Reference Materials for  $\delta^{18}\text{O}$  versus VSMOW Using Various on-Line High-

- Temperature Conversion Techniques. *Rapid Communications in Mass Spectrometry* **2009**, *23* (7), 999–1019.
- (35) Michalski, G.; Scott, Z.; Kabling, M.; Thiemens, M. H. First Measurements and Modeling of  $\Delta^{17}\text{O}$  in Atmospheric Nitrate. *Geophys. Res. Lett.* **2003**, *30* (16).
- (36) Grinfeld, D.; Aizikov, K.; Kreutzmann, A.; Damoc, E.; Makarov, A. Phase-Constrained Spectrum Deconvolution for Fourier Transform Mass Spectrometry. *Anal. Chem.* **2017**, *89* (2), 1202–1211.
- (37) Silva, S. R.; Kendall, C.; Wilkison, D. H.; Ziegler, A. C.; Chang, C. C. Y.; Avanzino, R. J. A New Method for Collection of Nitrate from Fresh Water and the Analysis of Nitrogen and Oxygen Isotope Ratios. *J. Hydrol.* **2000**, *228* (1), 22–36.
- (38) Wang, B.; Zheng, S.; Huang, Y.; Wang, Y.; Zhu, Z.; Ma, R.; Zhao, Y.; Yin, X.; Su, J.; Xiong, J.; Zhang, B.; Zhou, Y. Novel GC/Py/GC/IRMS-Based Method for Isotope Measurements of Nitrate and Nitrite. I: Converting Nitrate to Benzyl Nitrate for  $\delta^{18}\text{O}$  Analysis. *Anal. Chem.* **2020**, *92* (18), 12216–12225.
- (39) Owen, L. J.; Keevil, B. G. Testosterone Measurement by Liquid Chromatography Tandem Mass Spectrometry: The Importance of Internal Standard Choice. *Ann. Clin. Biochem.* **2012**, *49* (Pt 6), 600–602.
- (40) Graham, R. L. J.; Kalli, A.; Smith, G. T.; Sweredoski, M. J.; Hess, S. Avoiding Pitfalls in Proteomics Sample Preparation. *Biomacromol. Mass Spectrom* **2011**, *2* (4), 261–271.
- (41) Meier, F.; Geyer, P. E.; Virreira Winter, S.; Cox, J.; Mann, M. BoxCar Acquisition Method Enables Single-Shot Proteomics at a Depth of 10,000 Proteins in 100 Minutes. *Nat. Methods* **2018**, *15* (6), 440–448.
- (42) Tang, L.; Kebarle, P. Dependence of Ion Intensity in Electrospray Mass Spectrometry on the Concentration of the Analytes in the Electrosprayed Solution. *Anal. Chem.* **1993**, *65* (24), 3654–3668.
- (43) Guo, W.; Granger, J.; Sigman, D. M. Nitrate Isotope Fractionations during Biological Nitrate Reduction: Insights from First Principles Theoretical Modeling; 2010; Vol. 2010, p PP34A – 08.
- (44) Meija, J.; Coplen, T. B.; Berglund, M.; Brand, W. A.; De Bièvre, P.; Gröning, M.; Holden, N. E.; Irrgeher, J.; Loss, R. D.; Walczyk, T.; Prohaska, T. Isotopic Compositions of the Elements 2013 (IUPAC Technical Report). *Pure Appl. Chem.* **2016**, *88* (3), 293–306.

## Tables

<b>Table 1. Isotopologs of nitrate</b>			
<b>Mass range</b>	<b><i>m/z</i></b>	<b>abundance [ppm]</b>	<b>isotopolog</b>
<b>M0</b>	<b>61.9884</b>	<b>989,242</b>	<b><math>^{14}\text{N}^{16}\text{O}_3</math></b>
<b>M+1</b>	<b>62.9854</b>	<b>3,637</b>	<b><math>^{15}\text{N}^{16}\text{O}_3</math></b>
	<b>62.9926</b>	<b>1,127</b>	<b><math>^{14}\text{N}^{17}\text{O}^{16}\text{O}_2</math></b>
<b>M+2</b>	63.9896	4.1	$^{15}\text{N}^{17}\text{O}^{16}\text{O}_2$
	<b>63.9926</b>	<b>5951</b>	<b><math>^{14}\text{N}^{18}\text{O}^{16}\text{O}_2</math></b>
	63.9968	0.4	$^{14}\text{N}^{17}\text{O}_2^{16}\text{O}$
<b>M+3</b>	<b>64.9897</b>	<b>21.9</b>	<b><math>^{15}\text{N}^{18}\text{O}^{16}\text{O}_2</math></b>
	64.9938	<0.1	$^{15}\text{N}^{17}\text{O}_2^{16}\text{O}$
	<b>64.9968</b>	<b>4.5</b>	<b><math>^{14}\text{N}^{17}\text{O}^{18}\text{O}^{16}\text{O}</math></b>
	65.0010	<0.1	$^{14}\text{N}^{17}\text{O}_3$
<b>M+4</b>	65.9939	<0.1	$^{15}\text{N}^{17}\text{O}^{18}\text{O}^{16}\text{O}$
	<b>65.9969</b>	<b>11.9</b>	<b><math>^{14}\text{N}^{18}\text{O}_2^{16}\text{O}</math></b>
	65.9981	<0.1	$^{15}\text{N}^{17}\text{O}_3$
	66.0011	<0.1	$^{14}\text{N}^{17}\text{O}_2^{18}\text{O}$
<b>M+5</b>	66.9939	<0.1	$^{15}\text{N}^{18}\text{O}_2^{16}\text{O}$
	66.9981	<0.1	$^{15}\text{N}^{17}\text{O}_2^{18}\text{O}$
	67.0011	<0.1	$^{14}\text{N}^{17}\text{O}^{18}\text{O}_2$
<b>M+6</b>	67.9981	<0.1	$^{15}\text{N}^{17}\text{O}^{18}\text{O}_2$
	68.0011	<0.1	$^{14}\text{N}^{18}\text{O}_3$
<b>M+7</b>	68.9981	<0.1	$^{15}\text{N}^{18}\text{O}_3$

Isotopolog abundances are based on stochastically distributed isotope abundances corresponding to the primary standards AIR N<sub>2</sub> and VSMOW:  
 $^{14}\text{N} = 99.6337 \%$ ,  $^{15}\text{N} = 0.3663 \%$ ,  
 $^{16}\text{O} = 99.76206 \%$ ,  $^{17}\text{O} = 0.03790 \%$ ,  
 $^{18}\text{O} = 0.20004 \%$ .<sup>44</sup> The isotopologs in bold were measured in this study.



**Table 2. Flow injection of USGS reference materials,  $\delta$ -values in ‰ ( $\pm 1$  SD, n = 3)<sup>a</sup>**

Sample	$\delta^{15}\text{N}_{\text{Air}}$ ESI <sup>a</sup>	$\delta^{15}\text{N}_{\text{Air}}$ IRMS <sup>d</sup>	$\delta^{18}\text{O}_{\text{VSMOW}}$ ESI <sup>a</sup>	$\delta^{18}\text{O}_{\text{VSMO}}$ W IRMS <sup>d</sup>	$\delta^{17}\text{O}_{\text{VSMOW}}$ ESI <sup>a</sup>	$\delta^{17}\text{O}_{\text{VSMOW}}$ IRMS <sup>d</sup>	$\Delta^{17}\text{O}$ ESI <sup>a</sup>	$\Delta^{17}\text{O}$ IRMS <sup>d</sup>
<i>“with M0”: denominator is <math>^{14}\text{N}^{16}\text{O}_3</math></i>								
USGS35	2.6 $\pm$ 0.5	2.7	56.8 $\pm$ 0.1 <sup>c</sup>	56.8	51.1 $\pm$ 0.5	51.1	21.6 $\pm$ 0.4	21.6
USGS32	(178.0 $\pm$ 0.1) <sup>b</sup>	180	25.2 $\pm$ 0.3 <sup>c</sup>	25.3	13.0 $\pm$ 0.3	13.0	-0.1 $\pm$ 0.3	-0.2
USGS34	-2.2 $\pm$ 0.2	-1.8	(-28.1 $\pm$ 0.3) <sup>b,c</sup>	-27.8	(-16.8 $\pm$ 0.4) <sup>b</sup>	-14.8		
<i>“without M0”: denominator is <math>^{15}\text{N}^{16}\text{O}_3</math></i>								
USGS35			56.8 $\pm$ 0.4	56.8	51.5 $\pm$ 0.5	51.1	21.9 $\pm$ 0.4	21.6
USGS32			(28.0 $\pm$ 0.3) <sup>b</sup>	25.3	(14.4 $\pm$ 0.4) <sup>b</sup>	13.0		
USGS34			-27.7 $\pm$ 0.3	-27.8	-15.0 $\pm$ 0.1	-14.8	-0.6 $\pm$ 0.2	-0.3

<sup>a</sup> Values are averages of analyses done on 3 different days, with 4 replicates each. See also SI.

<sup>b</sup> USGS32 or USGS34 in combination with N11 were used for 2-point calibration (values in parentheses are the pre-scaled 1-point calibration data). Average  $\delta$  scale correction factors were 1.012 ( $\delta^{15}\text{N}$ ), 0.933 ( $\delta^{17}\text{O}$ ) for “with M0”, and 1.015 ( $\delta^{18}\text{O}$ ), 1.010 ( $\delta^{17}\text{O}$ ) for “without M0”.

<sup>c</sup>  $\delta^{18}\text{O}$  values in the “with M0” experiment were shifted by -0.36 ‰ relative to N11 (see text for discussion).

<sup>d</sup> See Table S2 in SI ( $\text{KNO}_3$ ,  $\text{NaNO}_3$  data).

**Table 3. Flow injection analyses of AgNO<sub>3</sub> from desert salt leachates,  $\delta$ -values in ‰ ( $\pm 1$  SD, n = 3)**

Sample	$\delta^{15}\text{N}_{\text{Air}}$ <i>ESI</i> <sup>a</sup>	$\delta^{15}\text{N}_{\text{Air}}$ <i>IRMS</i> <sup>c</sup>	$\delta^{18}\text{O}_{\text{VSMOW}}$ <i>ESI</i> <sup>b</sup>	$\delta^{18}\text{O}_{\text{VSMOW}}$ <i>IRMS</i> <sup>c</sup>	$\delta^{17}\text{O}_{\text{VSMOW}}$ <i>ESI</i> <sup>b</sup>	$\delta^{17}\text{O}_{\text{VSMOW}}$ <i>IRMS</i> <sup>c</sup>	$\Delta^{17}\text{O}$ <i>ESI</i> <sup>b</sup>	$\Delta^{17}\text{O}$ <i>IRMS</i> <sup>c</sup>
Atacama (N14362)	-0.5 $\pm$ 0.3	-0.2	54.3 $\pm$ 0.2	54.3	49.5 $\pm$ 0.2	49.4	21.3 $\pm$ 0.1	21.2
Antarctica (N17499)	-16.6 $\pm$ 0.1	-16.4	81.0 $\pm$ 0.2	80.8	73.6 $\pm$ 0.6	73.6	31.5 $\pm$ 0.5	31.6
Death Valley (N13315)	3.2 $\pm$ 0.3	2.9	nd	nd	nd	nd	nd	nd

N11 was used as a lab standard. Applied  $\delta$  scale correction factors for 2-point calibration were 1.012 ( $\delta^{15}\text{N}$ ), 0.998 ( $\delta^{18}\text{O}$ ) and 0.980 ( $\delta^{17}\text{O}$ ) (see Table S1).

<sup>a</sup> “with M0”

<sup>b</sup> “without M0”, denominator is  $^{15}\text{N}^{16}\text{O}_3$

<sup>c</sup> See Table S2 in SI (AgNO<sub>3</sub> data).

**Table 4. Dual inlet ‘dilute and shoot’ analyses of environmental samples,  $\delta$ -values in ‰ (NO<sub>3</sub><sup>-</sup> = 1  $\mu$ M; n = 1; “with M0”)**

Sample	$\delta^{15}\text{N}_{\text{Air}}$ <i>ESI</i>	$\delta^{15}\text{N}_{\text{Air}}$ <i>IRMS</i> <sup>f</sup>	$\delta^{18}\text{O}_{\text{VSMOW}}$ <i>ESI</i> <sup>d</sup>	$\delta^{18}\text{O}_{\text{VSMOW}}$ <i>IRMS</i> <sup>f</sup>	$\delta^{17}\text{O}_{\text{VSMOW}}$ <i>ESI</i> <sup>d</sup>	$\delta^{17}\text{O}_{\text{VSMOW}}$ <i>IRMS</i> <sup>f</sup>	NO <sub>3</sub> <sup>-</sup> <i>mmol/L</i> <sup>e</sup>	Cl <sup>-</sup> <i>mmol/L</i> <sup>e</sup>
Atacama (N14362) <sup>a</sup>	-0.8	0.0	54.9	55.1	54.8	49.8	44	82
Death Valley (N13315) <sup>a</sup>	3.4	3.5	25.5	24.2	20.6	20.7	57	nd
Death Valley (N14685) <sup>a</sup>	-0.6	-0.8	33.9	34.3	30.3	30.8	24	329
Namibia (N16854) <sup>a</sup>	8.2	9.1	28.8	28.6	19.6	21.0	36	3689
Antarctica (N17499) <sup>a,c</sup>	-16.1	-16.2	83.6	82.5	78.8	74.5	86	nd
UAE Sabkha (N13825) <sup>a</sup>	11.6	10.6	32.6	32.2	23.9	25.5	173	6974
New Mexico groundwater (N13993) <sup>b</sup>	8.4	6.8	-0.5	0.7	1.0	0.4	0.5	17.3
California groundwater (N17008) <sup>b</sup>	8.9	7.7	3.4	3.6	4.6	3.3	1.0	0.4
New York groundwater (N13819) <sup>b</sup>	5.7	7.3	2.8	3.5	0.0	1.8	0.5	0.3
Maryland groundwater (N10177) <sup>b</sup>	1.8	2.7	2.2	1.5	-0.4	0.8	1.3	0.4

<sup>a</sup> Concentrated leachate and brine samples

<sup>b</sup> Fresh groundwater samples

<sup>c</sup> This dilution had NO<sub>3</sub><sup>-</sup> = 50  $\mu$ M

<sup>d</sup> Blank corrected using sample data (see text for discussion)

<sup>e</sup> NO<sub>3</sub><sup>-</sup> and Cl<sup>-</sup> concentrations are given for original samples before dilution.

<sup>f</sup> See Table S2 in SI.

**Table 5. Exploration of  $\delta$ -values (in ‰) derived from clumped isotopes**

Sample	$\delta^{15}\text{N}_{\text{Air}}$ via $\delta(15\text{N}18\text{O}/18\text{O})$	$\delta^{15}\text{N}_{\text{Air}}$ <i>expected</i>	$\delta^{18}\text{O}_{\text{VSMOW}}$ via $\delta(15\text{N}18\text{O}/15\text{N})$	$\delta^{18}\text{O}_{\text{VSMOW}}$ <i>expected</i>
USGS35	$3.2 \pm 0.5$	2.7	$56.6 \pm 0.5$	56.8
USGS32	$174.4 \pm 0.3$	180	$23.7 \pm 0.6$	25.3
USGS34	$-2.9 \pm 1.0$	-1.8	$-27.9 \pm 0.9$	-27.8

“Without M0”, not scale corrected, assuming stochastic distribution in N11.