A proof-of-principle study for $\delta^{15}N$ measurements of aqueous dissolved nitrate and nitrite with a modified LC-IRMS interface

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Abstract

Rationale:

The analysis of nitrogen isotopes in aqueous dissolved nitrate is an effective method for identifying pollution sources and offers the potential to study the nitrogen cycle. However, the measurement of nitrogen isotope signatures of nitrate still requires extensive sample preparation or derivatization.

Methods:

In this study, a modified commercially available liquid chromatography-isotope ratio mass spectrometer (LC-IRMS) interface is presented that enables automated measurement of $\delta^{15}N$ signatures from nitrate by online reduction of nitrate in two consecutive steps. First, vanadium(III)-chloride is used as a reducing agent to convert NO_3^- to N_xO_y under acidic conditions. The mix of nitrogen oxides is then transferred into a stream of helium and reduced to nitrogen (N_2) analysis gas via a hot copper reactor. Prior to the online conversion of aqueous

nitrate into elemental nitrogen, the sample was chromatographically separated from potential matrix effects on a PGC column.

Results:

Precision was achieved at a level below 1.4 ‰ by injecting 10 µL of 50 mg L^{-1} N, using five different nitrate standards and reference materials. These materials spanned a range of more than 180 ‰ in $\delta^{15}N$. To demonstrate the applicability of the method we measured water samples from an enrichment experiment, where isotopically enriched ammonium chloride was administered into a small river over the course of two weeks. In contrary to our expectation, the $\delta^{15}N$ values of river nitrate showed values between +0.4 ± 0.4 ‰ and +4.1 ± 0.3 ‰, varying over a small range of 3.7 ‰.

Conclusions:

Our study showed that the measurement of nitrate nitrogen isotope signatures with a modified LC-IRMS system is possible, but that further modifications and improvements would be necessary for a robust and user-friendly instrument.

1 Introduction

The main inorganic nitrogen species in rivers are ammonium, nitrite and nitrate. [1] Contamination of water by anthropogenic nitrate has become a global environmental concern and the measurement of nitrogen isotope ratios of nitrate is an effective method for identifying and differentiating between natural and anthropogenic nitrate sources and it provides opportunities to study the nitrogen cycle. However, established methods for determining the nitrogen isotopic composition of nitrate at natural abundance levels are demanding and often hinder rapid and reliable measurements required in water monitoring programs. A variety of methods is used for the determination of the nitrogen isotope signature of nitrate in water:

(i) The ion-exchange method represents the earliest developed approach and employs an anion-exchange column for the preconcentration of nitrate and nitrite. They are then removed from the column by hydrochloric acid and analyzed as silver nitrate and silver nitrite using an elemental analyzer. [2]

(ii) The denitrifier method uses bacterial strains with lacking nitrous oxide reductase activity to stop the denitrification of nitrate and nitrite at N_2O, which is purified and trapped prior to IRMS analysis. [3]

(iii) The cadmium azide method uses a cadmium sponge to reduce nitrate to nitrite in a first step and to further reduce nitrite to nitrous oxide prior to IRMS analysis. [4]

(iv) Another method describes the measurement of ^{15}N abundances of aqueous nitrate and nitrite by on-line reduction to NO with vanadium or titanium chloride and subsequent measurement with a membrane-inlet quadrupole MS (SPINS/MIMS) [5] , based on a continuous-flow mass spectrometry method developed in 1999. [6] This method has been improved over the years [7,8] and the latest iteration uses an IRMS system instead of a quadrupole MS to measure isotopic ratios directly. [9,10] Samples with a nitrogen concentration of 35 µmol L^{-1} N for nitrate can be measured and $\delta^{15}N$ values from standards are reported with an accuracy of less than 0.9 ‰.

(v) Wassenaar, Altabet et al. developed a method based on the reduction to N_2O by reduction with Ti(III) with subsequent cryogenic purification and detection by IRMS [11] or without purification by laser spectroscopy. [12] The method provides the simultaneous measurement of $\delta^{15}N$ and $\delta^{18}O$ by IRMS and $\delta^{17}O$ values by laser spectroscopy.

(vi) Recently, Hilkert et al. showed that stable isotope ratio analysis (SIA) of nitrate is possible using ESI Orbitrap. Nitrogen ($\delta^{15}N_{AIR}$), oxygen $\delta^{18}O_{VSMOW}$, and $\delta^{17}O_{VSMOW}$ isotope ratios can be measured simultaneously with a long-term precision of ≤ 0.4 ‰ for reference material and purified nitrate samples. [13]

In order to develop an LC-IRMS method for the measurement of nitrogen isotope ratios from organic compounds, one problem that had to be solved first was whether nitrogen species formed during oxidation of organic compounds with peroxydisulfate that can be converted into a measurable gas form such as N_2O, NO or N_2. [14,15] Our subsequent objective was therefore to develop a method for measurement of nitrogen isotope signatures of nitrate through an online conversion into gaseous nitrogen oxide species (N_xO_y) and subsequent reduction to elemental nitrogen, while maintaining the capability for low-volume injections of samples. Furthermore, the goal was to achieve a chromatographic separation of nitrate from the injection peak and potentially nitrite. Here, we present the initial findings of standard sample measurements conducted with the modified interface and discuss the method's limitations and possible options for further improvement and future applications. Additionally, we had the opportunity to test our system with samples from an enrichment experiment conducted in a small sandy river.

2 Materials and Methods

2.1 Instrumental setup

The instrument that we used in our initial proof-of-principle study was a modified commercially available LC IsoLink™ interface (Thermo Fisher Scientific Inc., Bremen, Germany) (see Figure 1). In a preliminary step (i), nitrate was chromatographically separated from the injection peak and potential matrix interferences on a porous graphitic carbon (PGC)-HPLC column. In a second step, nitrate was reduced by a $V(III)Cl_3$ solution to N_xO_y with nitric oxide (NO) being the main product formed. Nitrogen oxides were separated from the eluent by membrane pervaporation (iii). After Nafion™ drying (iv), N_xO_y and NO were reduced to N_2 gas by a copper-filled ceramic reduction tube in a furnace (v). [16,17] Furthermore, oxygen was scavenged during this step, which enhances the precision and longevity of the filament in the IRMS ion source. Following reduction to N_2, carbon dioxide was removed by a cryo trap with a slurry of acetone / dry ice (vi) before the N_2 entered the IRMS via an open split.

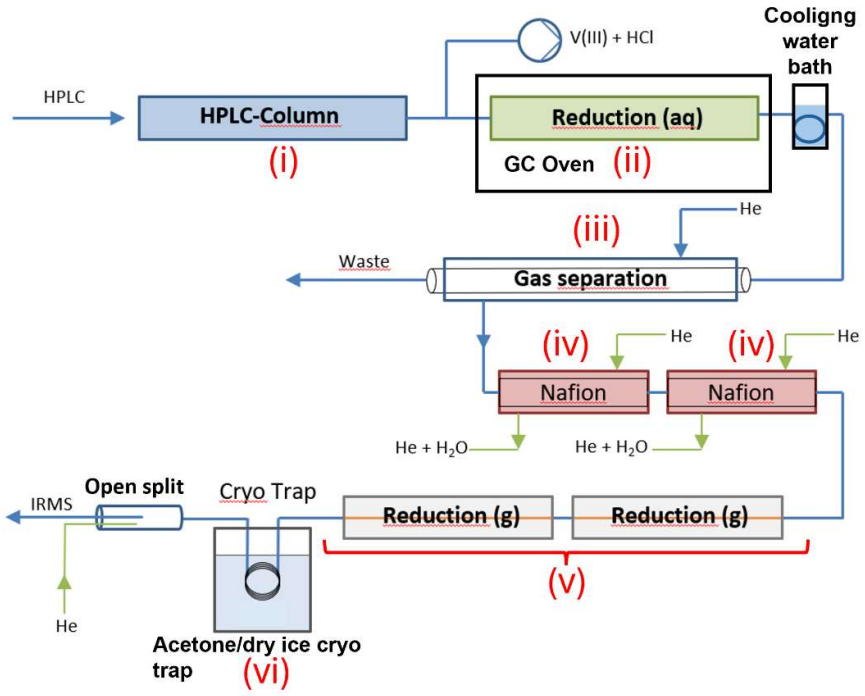


Figure 1 Instrumental setup of the modified LC-IRMS for the determination of $\delta^{15}N$ isotope values of nitrate and nitrite. (i) Chromatographic separation of nitrate by ion chromatography, (ii) reduction to N_xO_y, (iii) gas separation unit, (iv) gas drying by two nafion dryer units, (v) reduction reactor, (vi) aceton/dry ice cryo trap to trap CO_2. The nitrogen gas was introduced via an open split into the IRMS.

2.1 Chemicals and solutions

Vanadium(III)-chloride (97%, Merck, Darmstadt, Germany) was used to prepare solutions of 0.015 M V(III)Cl_3 in 0.3 M HCl (36.5 % – 38%, Alfa Aesar, Kandel, Germany). In order to preserve the HPLC pumps, which were constructed from stainless steel and were susceptible to high chloride concentrations, we did not utilize excessive amounts of reducing agents. Heated hydrochloric acid is highly corrosive and can irreparably damage the stainless-steel reactor in the LC-IRMS interface, therefore a deactivated silica capillary was used (see below). The reduction of nitrate to nitric oxide with vanadium(III)-chloride follows the chemical reaction [5]:

$$NO_3^- + 3V^{3+} + 4H^+ \rightarrow 3NO + 3V^{4+} + 2H_2 \qquad \text{(Eq. 1)}$$

The reduction of nitrate to nitrogen oxide requires the presence of three parts of vanadium(III) for every one part of nitrate. Therefore, a solution of 0.015 M VCl_3 is capable of reducing 0.005 M NO_3^-, which corresponds to a sample concentration of 140 mg L^{-1} NO_3^-. This concentration can be injected into the system and completely reduced if we consider that the mobile phase and reducing agents are mixed in a 1:1 ratio before they enter the reduction oven. The reduction of nitrate with vanadium(III) requires strongly acidic conditions at or below pH 1 [18], which was provided by the 0.3 M HCl solution in which the vanadium(III)-chloride was solved. For determination of conversion rates and selection of the acid see Supporting Information S 1 & 2. The solution was prepared with degassed Milli-Q water to avoid oxidation with dissolved oxygen and stored in a refrigerator for up to one week. The eluent was prepared by diluting sulfuric acid (95-97%, Merck, Darmstadt, Germany) to a concentration of 0.005 M H_2SO_4 or to a concentration required for the experiment.

All solutions were subjected to further purification under vacuum by a membrane pump (Vacuubrand GmbH & Co., Wertheim, Germany) and in an ultrasonic bath (Sonorex RK 100 Bandelin Electronic, Berlin, Germany) for a minimum of 15 minutes. They were continuously flushed with a small flow of helium (5.0) (Air Liquide, Oberhausen, Germany) throughout their use. The isotopic reference materials USGS 32, 34, and 35 (IVA Analysentechnik GmbH & Co. KG, Meerbusch, Germany) and nitrate in-house standards KNO_3 and $NaNO_3$ (>99%, Merck, Darmstadt, Germany) were measured on an Isoprime100 Elemental Analyzer (Elementar Analysensysteme GmbH, Langenselbold, Germany) for referencing purposes. The standard solutions typically contained 50 mg L^{-1} $N\text{-}NO_3^-$ (nitrate-N). One standard dilution series was prepared with concentrations of 10, 25, 50, 75, and 100 mg L^{-1} $N\text{-}NO_3^-$ to assess the concentration dependence of the system. For the determination of standard bulk isotope signatures of the by EA-IRMS see Supporting Information 3.

2.2 Experimental setup

147 A scheme of the modified system is depicted in Figure 1. The modified system based on the
148 commercially available LC-Isolink interface (Thermo Fisher Scientific, Bremen, Germany)
149 coupled to a DELTA V Advantage IRMS (Thermo Fisher Scientific, Bremen, Germany) for
150 continuous flow applications. Eluents and reducing agents were pumped with two separate
151 HPLC pumps (LPG-3400 SD and HPG-3200 SD, Thermo Fisher Scientific, Bremen,
152 Germany). The flow rates for the eluent and reducing agents were both set to 200 µL min^{-1}.
153 Nitrate injections were performed by an HTC PAL autosampler (CTC Analytics AG, Zwingen,
154 Switzerland) with different sizes of PEEK sample loops (5, 10, and 20 µL). The separation of
155 nitrate from the injection peak and other potential matrix effects was achieved with a
156 Hypercarb™ PGC column (2.1 x 100 mm, 3 µm, Thermo Fisher Scientific GmbH, Bremen,
157 Germany) and an eluent of 0.005 M H_2SO_4. Furthermore, the column temperature was elevated
158 to 80°C and maintained by an HT-HPLC 200 column oven (Scientific Instruments
159 Manufacturer GmbH, Oberhausen, Germany) in order to enhance control over retention times
160 and peak shape. The reducing agent was introduced to the eluent via a corrosion free, low dead
161 volume mixing chamber integrated into the commercial LC-Isolink interface and pumped into
162 a heated deactivated fused silica capillary (i.d. 0.32 mm; length 8 m, BGB Analytik, Böckten,
163 Switzerland) for reduction. The dimensions of the fused silica capillary result in a reactor
164 volume of approximately 0.64 mL and a residence time of the analytes that is longer than 1.5
165 minutes, with a combined flow rate of 0.4 mL min^{-1}. Heating was facilitated by a small selfmade
166 temperature-controlled GC oven, which was controlled by an Eurotherm 2216e microprocessor
167 (Schneider Electric Systems, Limburg, Germany) (see Supporting Information 2 and Figure
168 2B). The mobile phase was cooled after reduction by immersing the capillary (20 cm length,
169 0.32 i.d.) BGBAnalytik, Böckten, Switzerland) in a water bath at RT (23°C) before entering the
170 gas separation unit of the Isolink LC-IRMS interface. A small inline filter made of 4.9 mm
171 diameter, 10 µm pore size, PEEK™ encased (IVA Analysentechnik & GmbH, Meerbusch,
172 Germany) was installed before the gas separation unit to shield the three membranes from

potential non-soluble particles. The analytes were extracted and transported by a helium stream (Helium 5.0, Air Liquide, Oberhausen, Germany) of approximately 1–2 mL min^{-1} through the two Nafion™ membranes of the interface and into two subsequent heated copper reactors. Each reactor consisted of four individual copper wires (length 28 cm; o.d. 0.125 mm) twisted and inserted into a heated ceramic tube (length 320 mm; i.d. 0.5 mm) (both IVA Analytik, Meerbusch, Germany). This tube was inserted into a custom-made GC oven, which was controlled by two Jumo Itron16 (JUMO GmbH & Co. KG, Fulda, Germany) microprocessors and held at 650°C. The copper reactor and subsequent CO_2 trap, a slurry of acetone / dry ice (-78°C) for the LC-IRMS system, had been previously described in the literature [19] and served several purposes. The copper reactor acted as an oxygen scrubber, that increased the lifetime and precision of the filament. But it is of even greater significance in this context to note the ability of heated copper to reduce gaseous nitrogen oxide species to elemental nitrogen in accordance with the following equation:

$$y\,Cu + N_xO_y \rightarrow y\,CuO + \frac{x}{2}N_2 \qquad \text{(Eq. 2)}$$

The regeneration of the copper reactors was achieved by a stream of 3 % H_2 in He (Crystal Mixture, Air Liquide Düsseldorf, Germany) at 3 – 4 mL min^{-1}.

2.3 Test of reference gas stability

The intensity of the reference gas peaks was controlled by adjusting the gas pressure of the reference gas within the system using the pressure regulator of the interface. At the same time, the sample inlet split on the LC-Isolink interface was opened while a 0.05 M H_2SO_4 eluent and 0.015 M VCl_3 in 0.3 M HCl reducing agent were pumped at a rate of 200 mL min^{-1} each.

2.4 Enrichment experiment

The Rotbach River is a minor tributary of the Rhine in western Germany (51.5724°N 6.6871°E). The enrichment with heavy nitrogen (^{15}N) was carried out by using isotopically enriched

196 $^{15}NH_4Cl$ (Silantes, minimum 99 atom. % ^{15}N purity). It was diluted in 40 L distilled water for
197 each enrichment experiment. This tracer solution was released via a Duran glass Mariotte's
198 bottle (Schott, Mainz, Germany) which assured a consistent release of the solution independent
199 of the hydrostatic pressure within the bottle. In May and June 2021, a bottle of the labeled
200 material was exposed to the river in order to allow a constant portion to leach into the stream
201 over the course of six weeks. Water samples were collected at designated locations on a weekly
202 basis at distances of 50, 100, 200, 300, 500, 750, 1000, 1500, and 2000 meters downstream
203 from the administration point. Additionally, one sample was collected 50 meters upstream as a
204 reference. The samples were subsequently frozen and stored until further analysis. Given that
205 the modified LC-Isolink^{TM} interface was not suitable for direct injection and measurement of
206 nitrate from water samples, we proceeded to pre-concentrate the water samples after thawing
207 in a vacuum evaporator. This involved evaporating 50 mL of each water sample at 60°C and
208 under 50 mbar to approximately 1 mL, thereby enriching the samples by a factor of 50. Separate
209 spectroscopic measurements of ammonium, nitrite, and nitrate (Tabel S6) demonstrated that the
210 nitrate concentration in the river was approximately 7 mg L^{-1}, which corresponds to 1.58 mg L^{-1}
211 N. Enriching the nitrate concentration by vacuum evaporation 50-fold would result in a
212 concentration of ~80 mg L^{-1} N, which should fall within the previously determined
213 measurement range of the modified interface. Although the system still exhibits a non-linear
214 shift in $\delta^{15}N$ values and peak areas, this shift should not impede the ability to detect enriched
215 nitrogen isotope signatures. We proceeded to evaporate and measure a 1:50 diluted sample of
216 the in-house standard at an initial concentration of 50 mg L^{-1} N. This was done to ascertain the
217 impact of isotope fractionation effects during evaporation. Following evaporation, all samples
218 were filtered through 0.2 µm PTFE filters (FisherScientific, Schwerte, Germany), and 10 µL of
219 the resulting sample volume was directly injected into the system.

220 **3 Results and Discussion**

3.1 Stability and linearity of IRMS under measurement conditions

In order to assess the precision of the IRMS system for nitrogen isotope signatures under measurement conditions, ten consecutive reference gas peaks were injected with constant (stability) and increasing (linearity) signal intensities. Table S2 presents the average (Avg) and standard deviation (SD) of δ^{15}N values from ten reference gas peaks (denoted by *w*). These values were compared to linearity measurements without background signals, whereby the sample open split was turned off (denoted by *wo*). The SD of linearity measurements of nitrogen isotope signatures without any background signals under ideal IRMS conditions was 0.06 ‰ and increased to 0.41 ‰ conducting the same measurements with an active sample open split. The SD of stability measurements with an active sample open split (*w*) was 0.37 ‰. The main cause for the increasing SD of measurements with an active open split were outliers (peak no. 6 for linearity and peak no. 4 for stability measurements, marked with * in Table S2) throughout the complete series of ten reference gas injections. The cause for these outliers were small shifts in the m/z 29/28 ratios (see Supporting Information Figure S4) occasionally appearing throughout chromatograms. The shifts tend to be one-minute-long and, due to the nature of the interface to produce wide peaks, they might originate from the liquid phase of the system, possibly due to piston movement of the HPLC pumps. We took great care to manually check and avoid measurements where one of these shifts occurred under peaks of interest. Despite these interferences, the system performs sufficiently robust and is able to measure nitrogen isotope signatures of elemental nitrogen with a precision better than 0.5 ‰ over a peak area range of 4.6 to 21.3 Vs and possibly more precise if background shifts are avoided.

3.2 Separation of nitrate and nitrite from injection peak

Blank injections into the modified interface produced significant nitrogen peaks (Figure 2A), which also occurred with no reducing agents in the mobile phase and when the reactors were at room temperature. The measured δ^{15}N signatures of these peaks were between +2 and +8‰

against the nitrogen reference gas, which was set to zero. We therefore assumed that these blank peaks were either produced by dissolved elemental nitrogen in the sample or by switching the valve position of the 6-port valve from the autosampler. Switching the valves might have introduced small amounts of elemental nitrogen from the surrounding air into the eluent flow of the system. Separation of nitrate from the injection peak and nitrite was therefore crucial to avoid interferences and was achieved by a porous graphitic carbon (PGC) HPLC column (Hypercarb 100 x 2.1 mm, 5 µm, ThermoScientific). We used an elevated column temperatures at around 80°C and diluted sulfuric acid as anionic competitor to elute nitrate from the column. Without an anionic competitor, nitrate would be totally retained on the column. Sodium sulfate was used by Takeuchi et al. to separate inorganic anions including nitrate and nitrite on a PGC column [20], but we decided to use diluted sulfuric acid (0.005 M H_2SO_4) instead to keep the pH as low as possible for subsequent reduction (pH ~ 1). However, due to the broad peak widths produced in our modified interface and the LC-IRMS interface in general, efficient separation of nitrite and nitrate from the injection peak was not possible simultaneously (Figure 2B). Here, the HPLC flow was set to 200 µLmin^{-1} for both the eluent and reducing agent (0.03 M VCl_3 in 0.3 M HCl) and reactor column temperature was held at 55°C. Since the focus of this work was the measurement of nitrogen isotope signatures from nitrate, separation of nitrate from both blank and nitrite peaks was considered sufficient. Injecting a mixture of nitrate and nitrite (50 mgL^{-1} N each, 5 µL injection volume) under these flow conditions (Figure 2D) showed that nitrite was not fully separated from the injection peak. Reducing the eluent flow from 200 to 150 µLmin^{-1} and increasing the column temperature to 80°C (Figure 2C) almost separated nitrite (100 mg L^{-1} N, 5 µL injection volume) from the injection peak. Note that the injection volume was reduced from initially 10 µL (A and B) to 5 µL (Figure 2C and D) to reduce the load of nitrogen oxides on the copper reactor. The concentration of sulfate in the eluent, flow rates and column temperatures can be used to influence the retention time of nitrate, which is not only useful to control the to avoid possible matrix interferences or baseline fluctuations in

real samples. Testing different column parameters and materials might result in an improved performance of the system to separate both nitrate and nitrite from the injection peak.

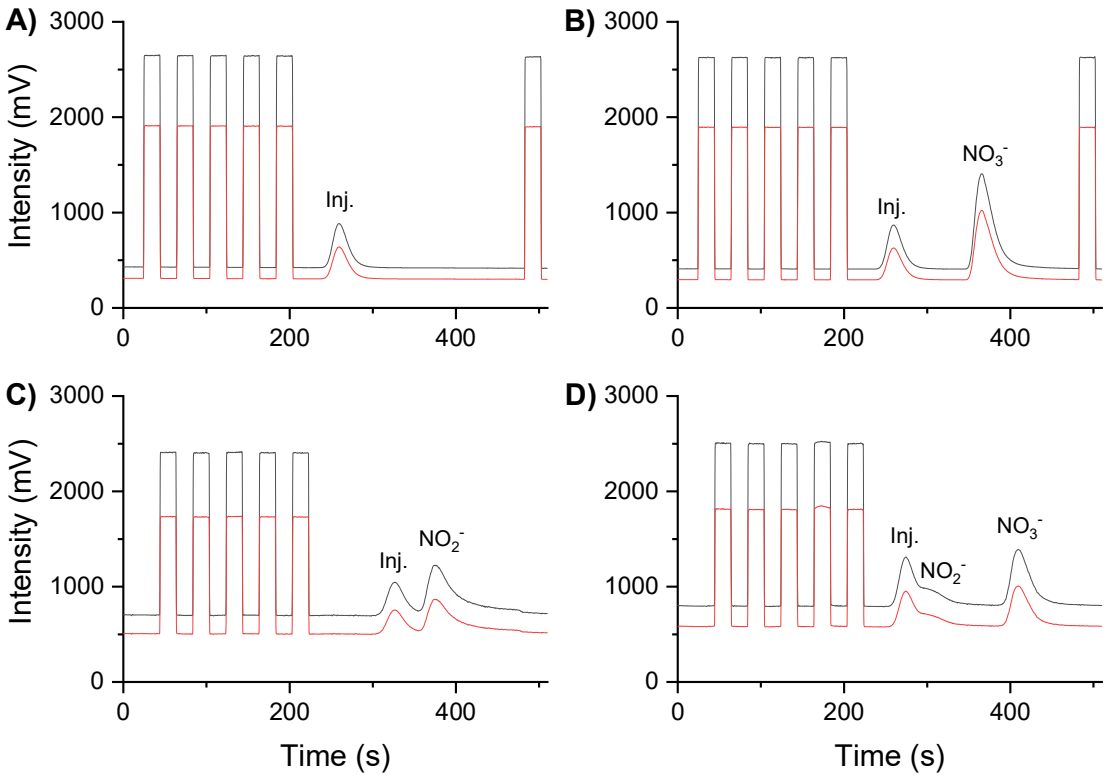


Figure 2 Chromatograms of blank (A), nitrate (B), nitrite (C) and a mix of both nitrate/nitrate (D) injections into the modified LC-IRMS interface for the measurement of nitrogen isotope signatures. Blank injections produce a measurable nitrogen peak (A).

3.3 Repeated nitrate measurements and reactor performance

To evaluate the ability of the copper reactor to reduce the high loads of nitrogen oxides coming from the modified LC-IRMS interface in addition to the oxygen loads coming from the eluent, we consecutively injected 5 µL of a 50 mgL^{-1} N solution of the USGS 34 international reference material on three following days. The copper reactor was reduced overnight in between measurement days with a 2 mL min^{-1} flow of 3 vol-% H_2 in He gas. The measured $\delta^{15}N$ values were constant for four to six injections, after which they started to decrease until the 20th to 25th injection (see Figure S5). This indicated that the reactor performance is stable for the first few injections, but quickly declines afterwards. Overnight regeneration of the reactor increased the measured $\delta^{15}N$ values on the next day, but not to the same values previously observed. This

showed that while regenerating the reactor is feasible, there might still be daily differences in reactor or system performance. Thus, frequent referencing through international or in-house standards, as typically required and practiced in CSIA, minimized the daily influence on the performance. Measuring nitrogen isotope ratios from nitrate with high precision throughout a day or for prolonged sample runs might therefore require a combination of two copper reactors in parallel, where one reactor is used for one or several injections of nitrate containing samples while the other is regenerated with a separate line of hydrogen gas.

3.4 Measuring different nitrogen reference materials with a wide range of isotopic signatures

Two international nitrate reference materials with certified nitrogen isotope signatures on natural abundance levels (USGS 34 and USGS 35) and one material enriched in its nitrogen isotope ratio (USGS 32) were utilized in this study. Additionally, two in-house nitrate standards (KNO_3 and $NaNO_3$) were calibrated on an EA-IRMS system with the three USGS reference materials. The referenced values of all five standard and reference materials are summarized in Table S1. We injected 10 µL of a nitrate solution containing 50 mg L^{-1} $N\text{-}NO_3^-$ of each material in triplicate and plotted the measured $\delta^{15}N$ values with all standard and reference materials (Figure 3A) and with materials on natural abundance levels (Figure 3B). The y-intercept of a linear regression curve between referenced and measured $\delta^{15}N$ values shows that measured $\delta^{15}N$ values are -8.1 and -8.2 ‰ lower than the referenced values, but a slope of 0.96 and 0.99 indicates that the differences between measured values of different materials are in good agreement to the differences of referenced values. As mentioned earlier, we occasionally observed small shifts in the *m/z* 29/28 ratio during measurements, which resulted in higher measured $\delta^{15}N$ values if the shifts overlapped with either a reference gas peak or a chromatographic peak and caused the observed difference. The reported values were produced throughout continuous measurements on two individual days and the copper reactors were regenerated overnight. The results are therefore also subject to isotope shifts over extended

measurement periods and to different copper reactor performance on individual sampling days. Addressing these issues might therefore further improve the precision and linearity of the regression. However, the reported values already show that the measurement of nitrogen isotope signatures from nitrate with the current setup was feasible on both natural and enriched abundance levels.

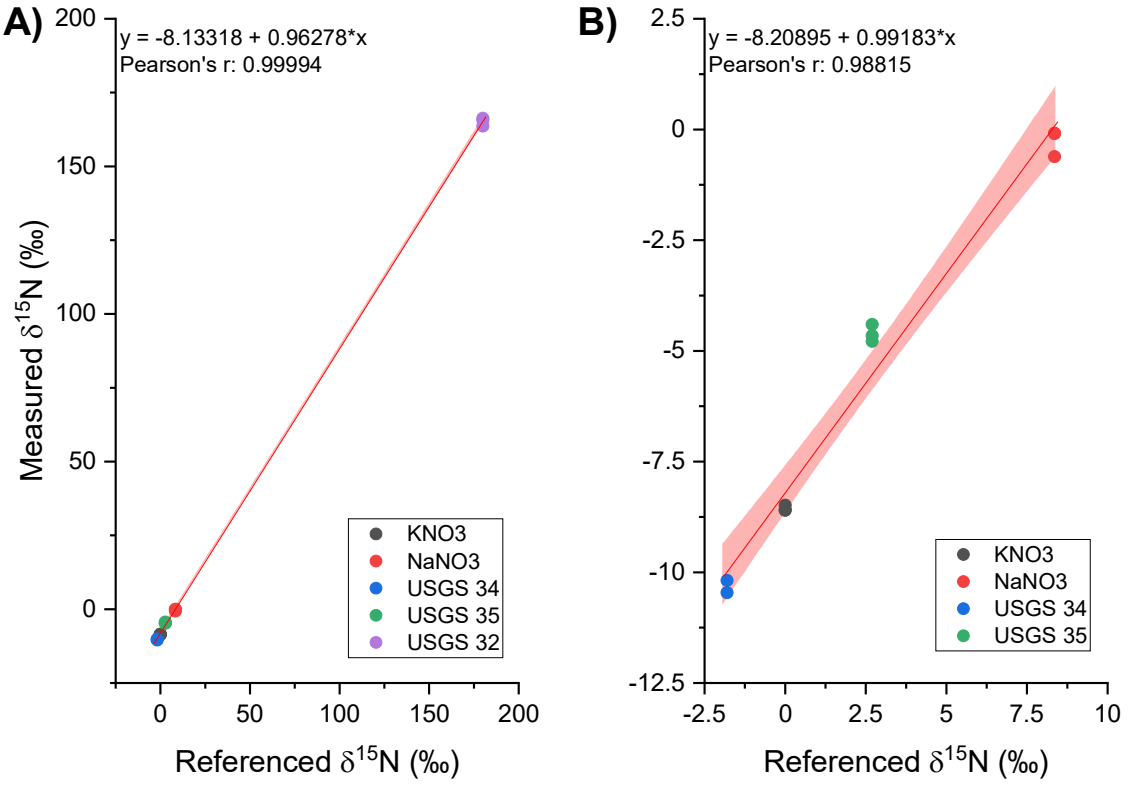


Figure 3 Differences between measured and referenced δ^{15}N values of reference materials and in-house standards were in good agreement. Since USGS 32 is an enriched reference material (A), it was removed for the linear regression (B) to decrease the range of δ^{15}N values to more natural abundance levels. Red line indicates linear regression curve and red area gives the 95% confidence interval. Samples were injected in triplicates.

3.5 Measuring different nitrate concentrations

To test the influence of nitrate concentration on nitrogen isotope signatures of nitrate, we prepared and measured a dilution series of $NaNO_3$ in-house standards. 10 µL injections of different N-$NaNO_3$ solution concentrations were done in triplicate and the measured δ^{15}N values and peak areas were plotted against the nitrogen concentration (Figure 4A & B). δ^{15}N values increase non-linear from -7.5 ± 1.4 ‰ to +1.8 ± 0.1 ‰ over a range of 10 to 100 mgL^{-1} N-$NaNO_3$ while peak areas simultaneously increase non-linear from 1.6 ± 0.0 Vs to 55.1 ± 0.3

Vs and are best described by a second order polynomial equation with a Pearson coeff. r of 0.998. The measured $\delta^{15}N$ values of 50 mg L^{-1} N-$NaNO_3$ was -0.4 ± 0.2 ‰. The lowest injected nitrate concentration of 10 mg L^{-1} N shows a noticeable increase in measurement uncertainty and the largest shift of $\delta^{15}N$ values. However, $\delta^{15}N$ values were stable once a concentration of 100 mg L^{-1} N-$NaNO_3$ was reached and peak areas showed a linear increase from 50 mg L^{-1} onwards. These concentrations are about 10 to 50 times higher than what is usually observed in water samples from areas affected by substantial agricultural activities, which results in nitrate concentrations between 10 and 50 mg/L^{-1} N-NO_3^-. [21] Especially, nitrate concentration in groundwaters or areas with limited agricultural activities were below the currently expected detection limits of the modified interface. As mentioned earlier, increasing the injection volume is a potential way of achieving the necessary analyte amount on the column if chromatographic separation is assured and the column is not overloaded. However, in our current set-up, sample enrichment prior to injection was necessary. Such an enrichment could be achieved by evaporation under vacuum (see next section) or by the use of anion exchange columns to preconcentrate nitrate, as described by the ion-exchange method. [2]

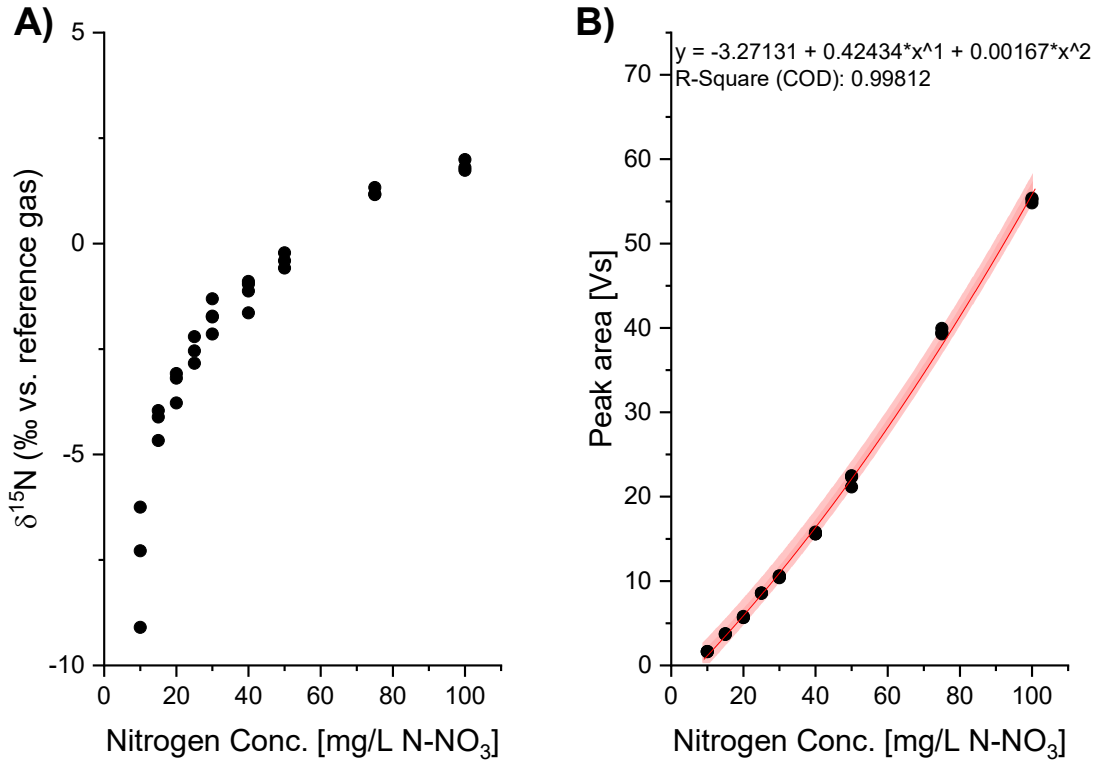


Figure 4 Injection of nitrate in different concentrations leads to a shift of measured $\delta^{15}N$ values (A) and a non-linear increase in peak areas (B). 10 µL $NaNO_3$ solutions from 10 to 100 mgL^{-1} N-NO_3 were injected in triplicate and the $\delta^{15}N$ values (‰) and peak area (Vs) measured. Peak integration was done by ISODAT software with the default settings of 0.2 and 0.4 mV s^{-1} start and end slope detection and

an individual background detection algorithm with a 5 second history. The increase in peak area over the concentration range is best described by a second polynomial equation with R-Square of 0.998.

3.6 Sample evaporation, blank samples and raw river water

In our study, we decided to test and employ sample evaporation to increase the nitrate concentration in real water samples. Injections of 10 µL MilliQ water (blank) into the system show blank peaks of dissolved nitrogen gas in the IRMS system after 260 s under the employed conditions, which had $\delta^{15}N$ values between +4 ‰ and +10 ‰ measured against the reference gas. No nitrate peak was visible in blank samples (Figure 5 bottom right). To test if evaporation of a nitrate solution leads to isotope fractionation, we diluted (1:50) standard solutions of 50 mg L^{-1} KNO_3 and 25 mg L^{-1} $NaNO_3$ and evaporated 50 mL down to 1 mL sample volume. Injecting 10 µL of the evaporated standard solutions and measuring the nitrogen isotope signature showed no significant difference in $\delta^{15}N$ values compared to non-evaporated standard samples (Welch's t-tests, $t_2 = -2.488$, $p = 0.124$ for $NaNO_3$ and $t_2 = 0.257$, $p = 0.821$ for KNO_3). The reported standard deviations for evaporated standard solutions were noticeable higher (1.7 ‰ for KNO_3 and 1.2 ‰ for $NaNO_3$) compared to normal standard solutions (0.2 ‰ for both KNO_3 and $NaNO_3$). Injecting raw river water from an isotopic enrichment experiment with $^{15}NH_4Cl$, which was not evaporated to pre-concentrate nitrate, also showed an injection peak after 265 s and no measurable nitrate peak (Figure 5 top right). The injection peak from ^{15}N-enriched raw water samples, however, exhibited a visible positive shift in *m/z* 29/28 ratios leading to an increased $\delta^{15}N$ value of +91.0 ± 2.5 ‰. The observed positive shift appeared not immediately with the beginning of the peak, where signal intensities start to rise, but shortly afterwards before the peak intensity reached its maximum. Spiking a raw water sample with nitrate in-house standards resulted in a nitrate peak after 376.2 s, while the positive isotope shift during the injection peak remained. The injection peaks of evaporated standard solutions further did not exhibit the unusual positive shift of the *m/z* 29/28 ratio observed in ^{15}N-enriched raw

water samples and the $\delta^{15}N$ values of the injection peaks remained under 10 ‰ and comparable to blank or regular standard solutions.

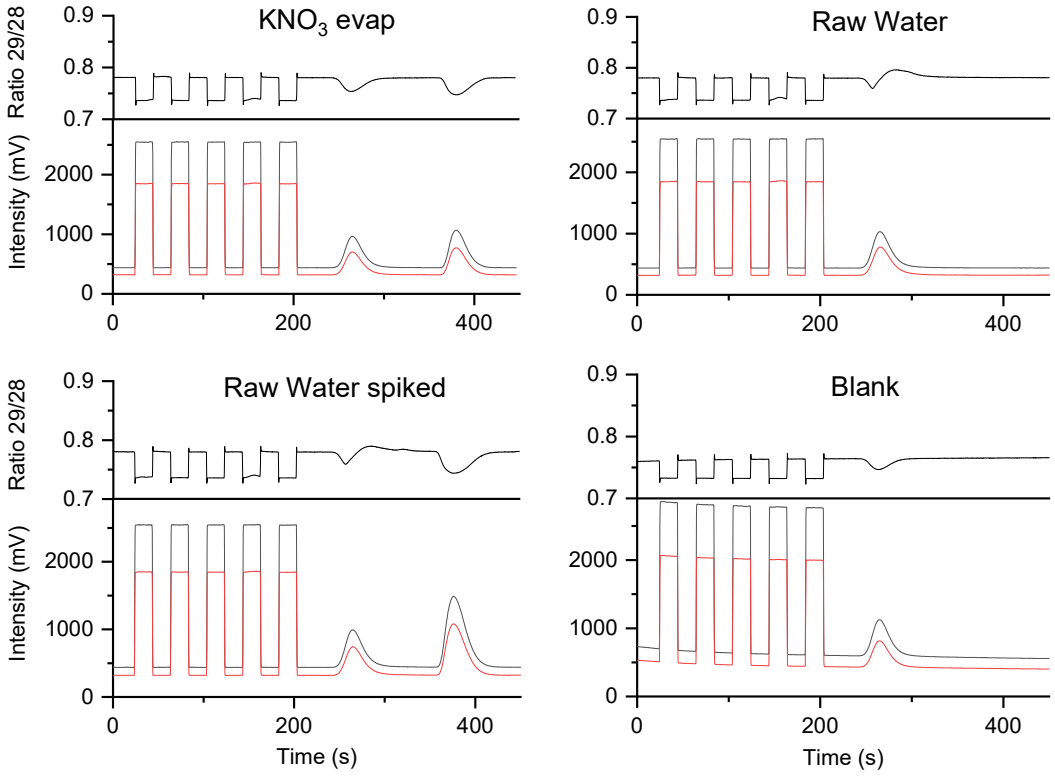


Figure 5: Chromatograms show measurements of evaporated KNO_3 standard solutions (top left), river water (top right), river water spiked with a nitrate standard (bottom left) and a blank sample of evaporated MilliQ water (bottom right). Evaporation overnight in a vacuum evaporator does not influence the *m/z* 29/28 ratio of either the nitrate peak (around 380 s) or injection peaks.

3.7 River samples from an enriched ammonium chloride administration experiment

The measured nitrate concentrations in the river Rotbach were between 6 to 8 mg L^{-1} (see Table S6) and the resulting nitrogen concentrations were around 1.2 mg L^{-1} $N\text{-}NO_3^-$, which could not be measured directly due to low sensitivity. Thus, we measured the nitrogen isotope signature of $N\text{-}NO_3^-$ from concentrated river water one and two weeks after administration of isotopically enriched ammonium chloride. In contrast to expectations, $\delta^{15}N$ values of river nitrate stayed between +0.4 ± 0.4 ‰ and +4.1 ± 0.3 ‰ in those two weeks, varying over a small range of 3.7

‰ (Table S5). No trends in $\delta^{15}N$ values were observed between sampling points or between sampling weeks (see Figure 6). After the second week, the reference point had a $\delta^{15}N$ value of +2.2 ± 1.7 ‰, which was well within the observed range of $\delta^{15}N$ values of nitrate downstream the administration point.

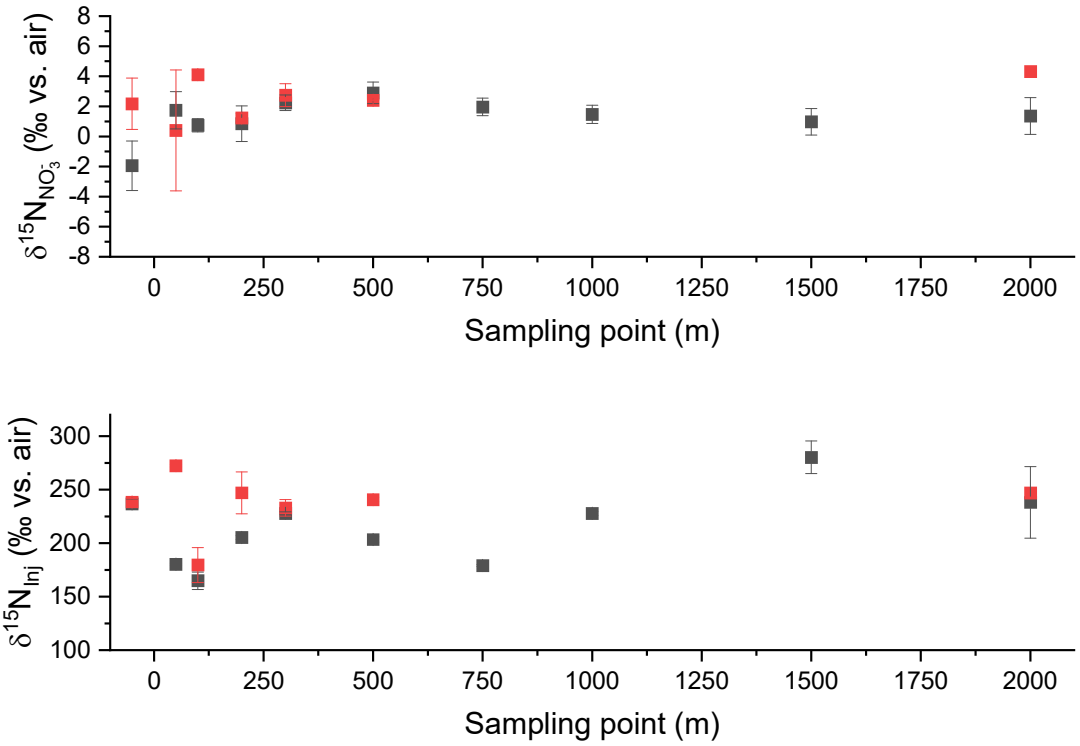
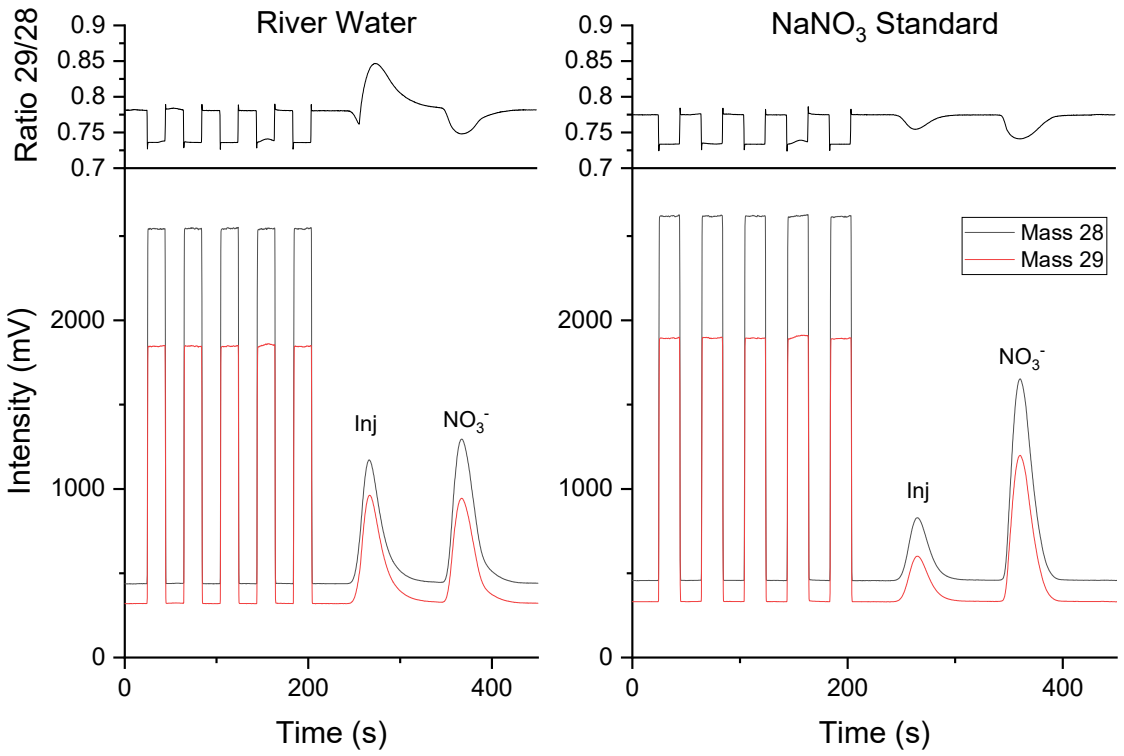


Figure 6: Nitrogen isotope signatures (‰ vs Air) of nitrate (NO_3^-, top) and the injection peak (Inj., bottom) from evaporated water samples of the river Rotbach after one week (grey boxed) and two weeks (red boxes) of introducing isotopically enriched ammonium chloride. Samples were taken up to 2000 m downstream of the administration point and one sample 50 m upstream as a reference.

The $\delta^{15}N$ values of the injection peaks for all samples during that time were much higher and ranged between +164.8 ± 8.1 ‰ and +272.4 ± 1.2 ‰, but also with no observable trend either between sampling points or weeks. The chromatograms of evaporated river water showed highly enriched compound eluting from the column right after the start of the injection peak (see Figure 7), as the *m/z* 29/28 ratio showed a strong positive shift shortly after the beginning of the chromatographic peak, which was comparable to what we also observed in non-evaporated water samples.



409

410 **Figure 7** Chromatogram of evaporated river water (left) and $NaNO_3$ standard solution at 50
411 mgL^{-1} $N\text{-}NO_3^-$ (right) showing separation of nitrate from the injection peak. Lines in the bottom
412 section represent signal intensities (mV) of m/z 28 (black) and 29 (red) for the determination
413 of $\delta^{15}N$ values by the IRMS. The upper sections show the ratio of *m/z* 29/28 intensities and
414 reveal an unusual swing in the injection peak for the river water sample, which is typically not
415 observed.

416

417 The isotope swing observed in raw water samples during the injection peak represents an
418 interesting result, leading to highly increased $\delta^{15}N$ values of the injection peak around ~90 ‰.
419 This indicates that either the atmospheric nitrogen in the river water is highly enriched in ^{15}N
420 or some enriched nitrogen oxide species are present in the sample, which are subsequently
421 reduced and measured as elemental nitrogen in the IRMS. The first possibility might be
422 unlikely, since the isotope swing does not completely overlap with the nitrogen injection peak
423 and has a small offset before the positive shift in *m/z* 29/28 ratio occurs. This would indicate
424 that the observed isotope swing during the injection peak of raw river water is caused by partial
425 coelution of isotopically enriched nitrite. The abundance of nitrite in river water is two orders

of magnitude lower compared to nitrate, which means that the isotope composition of nitrite in the water sample would be much higher than the measured δ^{15}N value of ~90 ‰, since it coelutes with the much more abundant nitrogen injection peak and typically has a δ^{15}N value of ~5 ‰. This is supported by measurements of evaporated river water, where the δ^{15}N value of the injection peak is even higher and reaches over +200 ‰, which would be a direct result of concentration of nitrite alongside nitrate in the sample material. In case of the administration study, our results suggest that concentration of nitrate in water samples via evaporation under vacuum is feasible without isotope fractionation and the measured δ^{15}N values of evaporated as well as non-evaporated nitrate standards are statistically not distinguishable.

Conclusions

In this study we presented a modified LC-IRMS interface for the measurement of nitrogen isotope signatures of nitrate from aqueous solutions. Chromatographic separation of nitrate from other nitrogen containing compounds and consecutive reduction to elemental nitrogen was achieved and nitrogen isotope signatures were measured from injections from up to 20 µL aqueous samples. We accomplished chromatographic separation of nitrate from blank peaks to increase accuracy and decrease matrix interferences.

Under current conditions, measurements of in-house standards and reference materials showed that the linearity of measured δ^{15}N values were in good agreement with both literature and referenced δ^{15}N values from an EA-IRMS system. Because of the early state of the modified system, this study can be interpretated as a proof of principle. Major challenges that should be addressed in the future include (i) reducing the nitrogen background from the eluent, (ii) modifying the copper reactors to operate in parallel to enable simultaneous regeneration of oxidized copper wires and (iii) reducing peak broadening by testing different column materials and optimizing flow conditions both in the liquid and gas phase. In addition, positioning of the switching valve heads into a nitrogen free gas atmosphere could reduce the amount of air

entering the system. These modifications will likely address the observed issues of low sensitivities as well as drifting isotope signatures over time. A separation and measurement of both nitrite and nitrate with one measurement might be possible if separation with other HPLC columns can be optimized.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authorship contribution statement

Tobias Hesse Methodology, Sampling Formal analysis, Investigation, Visualization, Writing - Original Draft. **Felix Niemann**: Sampling Formal analysis, Investigation, Review & Editing. **Shaista Khaliq**: Sampling Formal analysis, Review & Editing. **Daniel Köster**: Conceptualization, Methodology. **Julian Enss**: Sampling, Review & Editing. **Christian K. Feld**: Sampling, Review & Editing. **Milen Nachev**: Sampling, Review & Editing. **Klaus Kerpen:** Investigation. **Maik A. Jochmann:** Conceptualization, Methodology, Writing - Review & Editing, Supervision. **Torsten C. Schmidt**: Conceptualization, Methodology, Writing - Review & Editing Supervision.

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