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Experimental investigation of nitrogen and oxygen isotope fractionation in nitrate and nitrite during denitrification

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Abstract In batch experiments, we studied the isotope fractionation of nitrogen and oxygen during denitrification of two bacterial strains (*Azoarcus* sp. strain DSM 9056 and *Pseudomonas pseudoalcaligenes* strain F10). Denitrification experiments were conducted with succinate and toluene as electron donor in three waters with a distinct oxygen isotope composition. Nitrate consumption was observed in all batch experiments. Reaction rates for succinate experiments were more than six times higher than those for toluene experiments. Nitrogen and oxygen isotopes became progressively enriched in the remaining nitrate pool in the course of the experiments; the nitrogen and oxygen isotope fractionation varied between 8.6–16.2 and 4.0–7.3‰, respectively. Within this range, neither electron donors nor the oxygen isotope composition of the medium affected the isotope fractionation process. The experimental results provide evidence that the oxygen isotope fractionation during nitrate reduction is controlled by a kinetic isotope effect which can be quantified using the Rayleigh model. The isotopic examination of nitrite released upon denitrification revealed that nitrogen isotope fractionation largely follows the fractionation of the nitrate pool. However, the oxygen isotope values of nitrite are clearly influenced by a rapid isotope equilibration with the oxygen of the ambient water. Even though this equilibration may in part be due to storage, it shows that under certain natural conditions (re-oxidation of nitrite) the nitrate pool may also be indirectly affected by an isotope equilibration.

Keywords Nitrate · Nitrite · Denitrification · Nitrogen isotopes · Oxygen isotopes · Kinetic isotope fractionation · Isotope exchange

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Introduction

Especially in agricultural regions, dissolved nitrate is one of the most common contaminants that pose risk to shallow drinking water resources. The only natural process that may control the nitrate load of a drinking water body is bacterial denitrification (Kendall 1998). Furthermore, denitrification is recognized as one of the major processes for anaerobic biodegradation of a variety of organic compounds, including typical groundwater pollutants (for an overview see Widdel and Rabus 2001).

The reaction Eq. 1 summarizes the electron transfer processes during complete denitrification from dissolved nitrate to gaseous nitrogen.

$$2NO_3^- + 10e^- + 12H^+ \rightarrow N_2 + 6H_2O \quad (1)$$

Denitrification is a dissimilatory, respiration-coupled process carried out by several different enzymes, leading to different intermediates:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

Nitrate and nitrite reduction are catalyzed by the enzymes nitrate reductase (Nar) and nitrite reductase (Nir), respectively. Nitrate reductase is a membrane-bound enzyme located at the cytoplasmatic side of the membrane, which has been purified from several different denitrifiers (Zumft 1997). A second type of nitrate reductase located periplasmatically (Nap) is also widespread in bacteria, but presumably not linked to anaerobic respiration processes (Moreno-Vivián et al. 1999). Nitrate reduction in all known nitrate reductases is performed by a molybdenum cofactor (Zumft 1997; Moreno-Vivián et al. 1999). For dissimilatory nitrite reduction, two distinct enzymes are known: a tetraheme protein cytochrome cd_1 (NirS), and a copper-containing nitrite reductase (NirK) (Zumft 1997). The cytochrome cd_1 nitrite reductases are frequently present in the genera *Pseudomonas* (Zumft 1997) and *Azoarcus* (Song and Ward 2003; Rabus et al. 2005). The last steps of denitrification are catalyzed by NO reductase and nitrous oxide reductase (Zumft 1997).

For several decades, the dual isotope approach investigating both nitrogen and oxygen isotopic composition of nitrate has been successfully used to delineate nitrate sources, to reveal nitrate transport pathways, and to identify nitrogen transformation processes such as nitrification or denitrification in aquifers (e.g. Böhlke and Denver 1995; Aravena and Robertson 1998; Böhlke et al. 2002; Einsiedl et al. 2005; Einsiedl and Mayer 2006; Osenbrück et al. 2006), lakes (e.g. Knöller and Strauch 1999; Lehmann et al. 2003; Bozau et al. 2006), and marine environments (e.g. Brandes et al. 1998; Voss et al. 2001; Casciotti and McIlvin 2007).

While for most of the case studies nitrate was in the focus of the investigation, recent technical developments enable the isotopic examination of nitrite as well. The combination of the bacterial denitrifier method for a simultaneous determination of nitrogen and oxygen isotope values using micro-amounts of samples (Sigman et al. 2001; Casciotti et al. 2002) with chemical (Granger et al. 2006) or biological preparation techniques (Böhlke et al. 2007) allows the separate isotopic analyses of nitrate and coexisting nitrite.

For a reliable prediction of the hydrochemical evolution of a drinking water resource affected by nitrate input or the assessment of the overall natural attenuation potential of a contaminated aquifer where anaerobic biodegradation under nitrate-reducing conditions is occurring, a quantification of the denitrification process is essential. This quantification, using stable isotope ratios in concert with concentration data, is always based on isotopic enrichment factors. Isotope enrichment factors for denitrification reported so far are mostly based on field data (e.g. Böttcher et al. 1990; Mengis et al. 1999; Lehmann et al. 2003). Due to potential fluxes (often open system conditions), those enrichment factors may have a relatively high uncertainty. Only a very few experiments have been conducted in order to determine the enrichment factors for both oxygen and nitrogen during denitrification (Olleros 1983; Granger et al. 2008). This might be partially due to experimental problems arising from the nitrite accumulation during denitrification experiments. In particular, an incomplete separation of accumulated nitrite and residual nitrate may bias the experimental results. Furthermore, the experimental design has to take the reactivity of nitrite into account in order to avoid any re-oxidation.

It is generally agreed that denitrification causes a kinetic isotope effect on both nitrogen and oxygen isotopes (Mariotti et al. 1981; Chen and MacQuarrie 2005; Granger et al. 2008) and that the ratio of the enrichment factors for oxygen and nitrogen isotopes is between 0.5 and 1, as summarised by Chan and MacQuarrie in 2005 or by Granger et al. in 2008.

Analogously to sulfate, the oxygen isotope exchange between nitrate and the ambient water is extremely slow under environmental conditions typical for groundwater ecosystems. Accelerated isotope exchange between sulfate or nitrate and water only occurs under extreme environmental conditions such as low pH and/or elevated temperature (Lloyd 1968; Chiba and Sakai 1985; Böhlke et al. 2003). However, recent studies have shown that microbial reduction may speed up the isotope exchange via cell-internal intermediates so that the oxygen isotope fractionation during bacterial sulfate reduction is strongly influenced by an oxygen isotope equilibration with the ambient water (Brunner et al. 2005; Knöller et al.

2006). That fact led us to pose the question to what extent a potential oxygen isotopic exchange can affect the oxygen isotope evolution of the nitrate pool during its microbial reduction. Furthermore, the intention of this investigation was to examine the potential variability of the isotopic enrichment factors for nitrogen during denitrification related to diverse electron donors and the extents of microbial nitrite accumulation. This knowledge is essential for understanding the natural variation of isotope fractionation during denitrification and, eventually, for a field-scale quantification of the process.

Experimental settings and methods

Batch experiments

Altogether, nine different batch experiments were conducted in duplicates under an oxygen-free atmosphere using the bacterial strains *Pseudomonas pseudoalcaligenes* strain F10 (Martienssen and Schöps 1997) and *Azoarcus* sp. strain DSM 9056. *P. pseudoalcaligenes* was kindly provided by Marion Martienssen, Helmholtz Centre for Environmental Research, Dep. Hydrogeology, *Azoarcus* sp. DSM 9056 was obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. *P. pseudoalcaligenes* has been described to reduce nitrate without transient accumulation of nitrite (Martienssen and Schöps 1997). The *Azoarcus* strain reduces nitrate to nitrite, which is further reduced to N2 after a certain lag-phase (Dolfing et al. 1990). None of these strains was described for nitrate reduction to ammonium. While *P. pseudoalcaligenes* was only inoculated with succinate as electron acceptor, the experiments with the *Azoarcus* strain were conducted with two different electron donors: succinate and toluene. Both strains were cultivated in an anoxic mineral salt medium containing the following compounds (in g l^{-1}): KH_2PO_4, 0.1; NH_4Cl, 0.4; KCl, 0.4; Na_2SO_4, 0.2; $CaCl_2$, 0.1; $MgCl_2$, 0.5; KNO_3, 1.01; $NaHCO_3$, 2.52. The medium was completed by adding 5 ml l^{-1} anoxic vitamin solution (Pfennig et al. 1965) and 1 ml l^{-1} anoxic trace element solution 10 (DSMZ medium no. 320). Finally, the pH was adjusted to 7 using 2 M HCl. The media and solutions were always flushed with sterile nitrogen to remove oxygen after autoclaving or sterile filtering. Three mineral salt media were prepared using three waters of different oxygen-isotopic composition (H_2O-light = −31.2‰, H_2O-medium = −8.8‰, H_2O-heavy = 4.5‰ VSMOW, respectively). Each electron donor/bacterial strain combination experiment was carried out with each of the three isotopically different waters. Succinate (final concentration: 20 mM) was added by means of an anoxic stock solution (1 M). In order to avoid a toluene stress on the *Azoarcus* strain, only 5 µl toluene was initially added as pure compound. After 40 h, 15 µl of toluene was additionally given to each toluene degradation experiment with Azoarcus (AzuTol). Nitrate was given in concentrations between 10 and 20 mM. Each degradation experiment was performed in duplicate in a 250 ml bottle filled with 245 ml medium and inoculated with 5 ml bacterial culture taken from the early stationary phase of a culture grown on the same electron donor/acceptor combination used in the isotope experiment. For each degradation experiment, a single abiotic control bottle was set up which was prepared as described for the respective biotic variant, but autoclaved (20 min, 121°C) immediately after inoculation with bacteria.

From each of the 27 glass bottles (nine different experiments in duplicates and the respective sterile controls), two aliquots of samples (4 ml each) were taken at intervals of several hours. Samples were always taken inside an anaerobic glove box (gas atmosphere: 95% nitrogen, 5% hydrogen; Coy Laboratory Products Inc., USA) to exclude oxygen contaminations. Right after sampling, all samples were passed through sterile filters of 0.2 µm pore size under oxygen-free atmosphere in order to remove all biomass and stop the bacterial degradation of nitrate. After filtration, the samples were immediately transferred to a freezer and stored at −20°C until further processing. The storage time for all samples before isotope analyses was between 15 and 21 days.

Chemical and isotope analyses

For all samples, the content of dissolved nitrate and nitrite was determined by ion chromatography (IC-100, Dionex) from one of the 4 ml aliquots. A part of the second aliquot was used for the nitrogen and oxygen isotope measurement of the bulk samples containing both nitrate and nitrite. Prior to isotope

analysis, the bulk samples were freeze-dried and redissolved in water with the same oxygen isotopic composition ($\delta^{18}O = -8.6‰$ VSMOW) as the water used for dissolving the reference nitrates for isotope analysis. The amount of water used for redissolving the freeze dried samples was varied in such a way that the final N-concentration of each sample was ca. 0.3 mM. This was done to provide similar conditions for all experimental and reference samples with respect to the total amount of nitrate and to potential isotope exchange with the water in the sample vials.

The nitrite and nitrate fractions of sample solutions were separated using a common ion chromatography system (DX-100; DIONEX) connected to a conductivity detector. The isocratic separation was carried out with an analytical anion column (IONPAC AG14; DIONEX) working with 1.2 ml min^{-1} flow of 3.5 mM sodium carbonate/1.0 mmol l^{-1} sodium hydrogen carbonate eluent. Each nitrite fraction was collected under nitrogen gas atmosphere. Immediately after collection, the nitrite samples were freeze-dried. Prior to isotope analysis, the freeze dried samples were re-dissolved in defined amounts of de-ionized water with a constant oxygen isotope composition ($\delta^{18}O–H_2O = -8.60‰$ VSMOW).

The isotope analyses were conducted on a GasbenchII/delta V plus combination (Thermo) using the denitrifier method for a simultaneous determination of $\delta^{15}N$ and $\delta^{18}O$ in the measuring gas N_2O, produced by controlled reduction of sample nitrate/nitrite (Sigman et al. 2001; Casciotti et al. 2002). For calibration of nitrogen and oxygen isotope values of bulk samples as well as of nitrogen isotope values of nitrite samples, the reference nitrates IAEA-N3 ($\delta^{15}N$: +4.7‰ AIR; $\delta^{18}O$: +25.6‰ VSMOW) USGS32 ($\delta^{15}N$: +180‰ AIR; $\delta^{18}O$: +25.7‰ VSMOW), USGS 34, and USGS 35 ($\delta^{15}N$: +2.7‰ AIR; $\delta^{18}O$: +57.5‰ VSMOW) were used. To test for potential fractionation during chromatographic separation, solutions of IAEA-N3, USGS34, USGS35, and of two commercially available nitrites (KNO_2, $NaNO_2$, Merck) were used. The $\delta^{15}N$ and $\delta^{18}O$ values of the nitrites had previously been determined with an elemental analyzer/delta V plus (Thermo) and a TC/EA–delta XL plus (Thermo), respectively (KNO_2: $\delta^{15}N = -19.5‰$, $\delta^{18}O = 10.2‰$; $NaNO_2$: $\delta^{15}N = -22.6‰$, $\delta^{18}O = 11.8‰$). Pure NO_3 or NO_2 standard solutions covering a concentration range between 0.1 and 15 mmol l^{-1} as well as mixed nitrite/nitrate solutions with various mixing ratios (NO_3:NO_2 between 10:1 and 1:10) and concentrations (between 0.2 and 10 mmol l^{-1}) were injected into the chromatography column. The isotope analyses of the recovered fractions revealed that the isotopic composition of the nitrite was not affected by the chromatographic separation ($\Delta^{15}N$: −0.18… +0.24‰; $\Delta^{18}O$: −0.37… +0.32‰). However, systematic isotope fractionation appeared to occur in the nitrate fraction ($\Delta^{15}N$: +0.95… +3.55‰; $\Delta^{18}O$: −2.02… −4.17‰). Therefore, the isotopic composition of nitrate could not be measured directly but had to be calculated using the isotopic composition of the bulk sample and that of the nitrite fraction. Due to the oxygen isotope fractionation that occurs during the reduction of nitrate to nitrite, the calibration of $\delta^{18}O$ values of nitrite samples using nitrate reference materials would return oxygen isotope values of nitrite that are 25–30‰ too low (Casciotti et al. 2007). Therefore, for the exact calibration of nitrite–oxygen versus VSMOW nitrite reference materials have to be used. However, when the subtraction method is applied to compute the $\delta^{18}O–NO_3^-$ from the $\delta^{18}O$ of the bulk sample ($\delta^{18}O_{nitrate} = \delta^{18}O_{bulk}/X_{nitrate} - \delta^{18}O_{nitrite} * X_{nitrite}/X_{nitrate}$ with $X_{nitrite} + X_{nitrate} = 1$) and the bulk samples are calibrated using nitrate reference materials, the nitrite measurements have to be calibrated with nitrate reference materials as well (Casciotti et al. 2007).

According to the findings of Casciotti et al. (2007), the nitrite-water isotope equilibration at neutral pH proceeds within a matter of weeks even for frozen samples stored at −20°C, implying that the oxygen isotope ratio of nitrite rapidly approaches a steady state value determined by the isotope ratio of the ambient water. Therefore, no mechanistic conclusions can be drawn from the nitrite oxygen isotope data. However, the exact measurement of the $\delta^{18}O–NO_2^-$ is vital for calculating correct $\delta^{18}O–NO_3^-$ values from bulk sample measurements.

The relatively rapid isotopic equilibration between nitrite and ambient water raises concerns that the oxygen isotopic composition of nitrite reference materials and nitrite samples may be affected during the analytical procedure. To minimize the potential effect of oxygen isotope equilibration, periods between dissolution of the freeze dried samples or crystalline reference materials and the second freeze drying step following the chromatographic separation were kept as short as possible (less than 60 min).

Consequently applying that procedure, no influence of a potential nitrite–water equilibration was seen during the above mentioned test measurements using different concentration and mixing ratios of reference materials.

Nitrogen and oxygen isotope measurements were performed with an analytical error of the measurement better than ±0.3 and ±0.6‰, respectively. Results are reported in delta notation ($\delta^{15}N$, $\delta^{18}O$) as parts-per-thousand (‰) deviations relative to the standards AIR (for nitrogen) and VSMOW (for oxygen).

The enrichment factors for nitrogen and oxygen were calculated using the Rayleigh equation 2 as proposed by Mariotti et al. (1981)

$$\varepsilon = \frac{10^3 \ln \frac{10^{-3}\delta(NO_3^-)_{measured}+1}{10^{-3}\delta(NO_3^-)_{initial}+1}}{\ln\left[C(NO_3^-)_{measured}/C(NO_3^-)_{initial}\right]} \quad (2)$$

where ε stands for the isotopic enrichment factors for nitrogen and oxygen, δ stands for the $\delta^{15}N$ and $\delta^{18}O$ values, respectively, and C stands for the nitrate concentration.

Results and discussion

Nitrate reduction in batch experiments

Batch experiments were set up with succinate and toluene as electron donors and nitrate as sole electron acceptor. Any consumption of dissolved nitrate is related to bacterial nitrate reduction. As shown in Figs. 1, 2 and 3a, b, an almost complete nitrate consumption (between 80 and 100%) was observed in all batch experiments. However, consumption rates differed considerably between the experiments with different bacterial strains and electron donors. The fastest nitrate consumption was evident in the experiments with *P. pseudoalcaligenes* using succinate as electron donor (PSuc experiments, Fig. 3). Within less than 20 h the nitrate was completely removed. A comparable nitrate reduction rate was observed in the experiments with *Azoarcus* sp. and succinate as electron donor (AzoSuc experiments, Fig. 1). During these experiments, an almost total consumption of nitrate (up to 99.8%) was reached after 25–28 h. A significantly lower reaction rate was present in the experiments with *Azoarcus* sp. and toluene as electron donor (AzoTol experiments, Fig. 2). After 175 h, between 80 and 88% of the original nitrate were reduced.

A temporary accumulation of dissolved nitrite was observed in all batch experiments (Figs. 1b to 3b). The potential of *P. pseudoalcaligenes* for accumulating nitrite during proceeding denitrification was supposedly very low (Martienssen and Schöps 1997) which was one reason for selecting this strain for our experiments. Nevertheless, *P. pseudoalcaligenes* accumulated nitrite in concentrations up to 3.7 mmol l^{-1} (Fig. 3b). However, the nitrate concentration was not significantly exceeded by the accumulated nitrite in the course of those experiments (maximum $C\text{–}NO_2^-/C\text{–}NO_3^- = 1.2$). The nitrite accumulation of *Azoarcus* sp. is more pronounced. During the experiments using toluene as electron donor, a maximum nitrite accumulation of more than 6 mmol l^{-1} was observed (ca. 45% of initial N_{total}). The concentration of coexisting dissolved nitrate was temporarily exceeded by more than 4 mmol l^{-1} (maximum $C\text{–}NO_2^-/C\text{–}NO_3^- = 2.3$). The temporal development of nitrite and nitrate concentrations in the course of the experiments with succinate as electron donor was similar to that in the experiments with toluene. The maximum nitrite concentration (4.1 mmol l^{-1}) temporarily reached 45% of the initial total nitrogen concentration; the maximum ratio $C\text{–}NO_2^-/C\text{–}NO_3^-$ was 2.2.

Generally, the accumulation of nitrite during the batch experiments for both strains is obviously determined solely by the specifics of the enzymes catalyzing nitrate and nitrite reduction. In *Pseudomonas* and *Azoarcus* strains, nitrite reduction is usually catalyzed by a cytochrome cd_1 nitrite reductase (NirS) (Rabus et al. 2005; Song and Ward 2003; Zumft 1997). Dissimilatory nitrate reduction under anoxic conditions is carried out in most bacteria by a membrane-bound nitrate reductase (NAR).

Nitrogen isotope fractionation during denitrification

Even though the complete reduction of dissolved nitrate to gaseous nitrogen involves several intermediate nitrogen oxide species, it is generally agreed that the remaining nitrate pool during denitrification is affected by a straightforward kinetic enrichment of ^{15}N (e.g. Kendall 1998; Chen and MacQuarrie 2005).

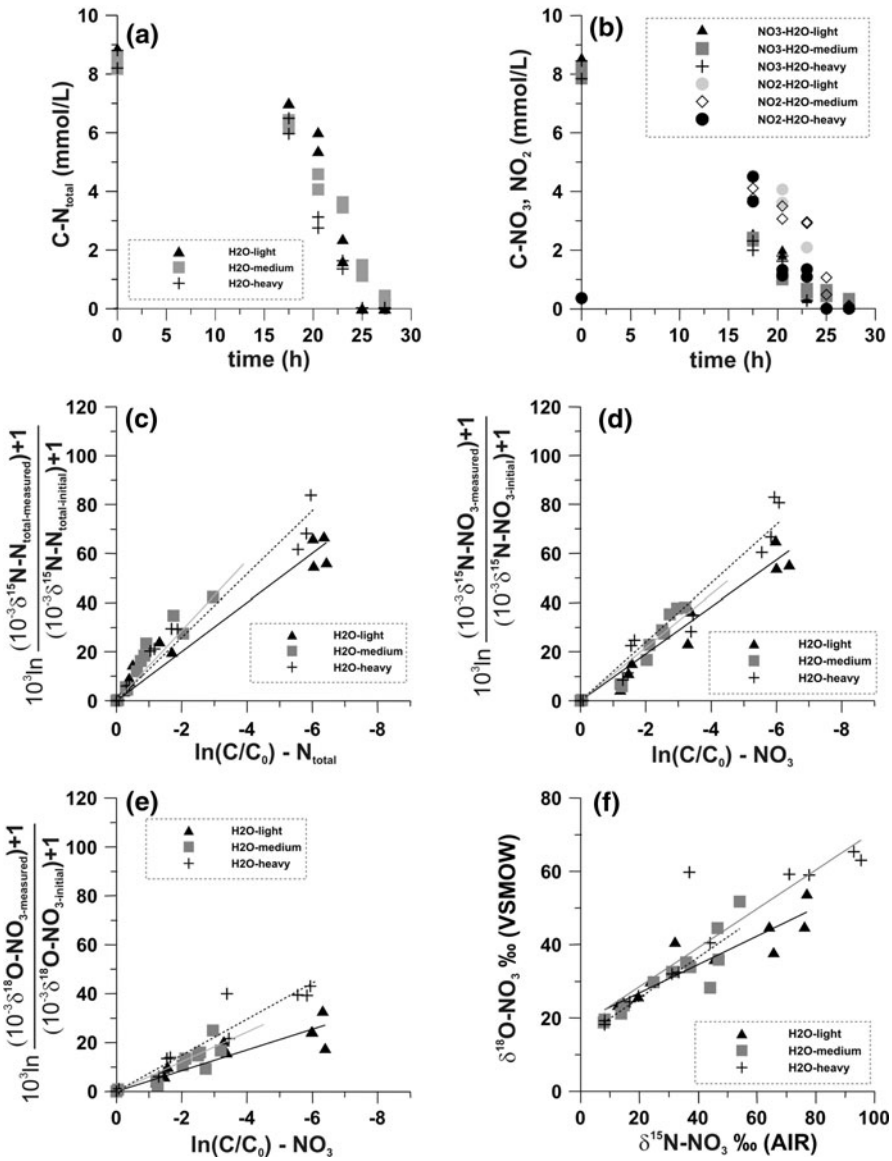

Fig. 1 Temporal development of total N (**a**), as well as nitrate and nitrite (**b**) concentrations for batch experiments with *Azoarcus* sp. using succinate as electron donor and three isotopically different waters. Furthermore, the figure shows the correlations between the residual total N pool (nitrate + nitrite) and normalized nitrogen isotope ratios (according to Eq. 2) of the total N pool (**c**), between the residual nitrate and the normalized nitrogen (**d**) and oxygen (**e**) isotope ratios of nitrate, and between $\delta^{15}N–NO_3$ and $\delta^{18}O–NO_3$ values (**f**)

This kinetic enrichment can be described by an Eq. 2 proposed by Mariotti et al. (1981). The crucial parameter determining the isotopic enrichment during nitrate reduction is the enrichment factor $^{15}\varepsilon$. The exact knowledge of the enrichment factor is essential for the characterization and the quantitative assessment of nitrate reduction as well as for the estimation of processes affecting the isotope fractionation in aquifers, such as mixing or secondary nitrification, that may superimpose the isotope fractionation of the original nitrate reduction process.

An overview of isotope enrichment factors estimated from several culture experiments and from case studies in natural environments is presented by Lehmann et al. (2003). The large variation range for $^{15}\varepsilon$, from −5 to −40‰, suggests a strong dependency of the enrichment factor on several experimental and environmental conditions as well as reaction pathways and involved enzymes. The extent of $^{15}\varepsilon$ for nitrate mainly depends on the first irreversible step of the biochemical reaction mechanism of nitrate reduction, but is also controlled by rate-limiting processes preceding the enzymatic reaction (e.g. uptake and transport of a substrate to the reactive site of enzyme, binding of the substrate to form enzyme–substrate complexes), which can significantly mask isotope

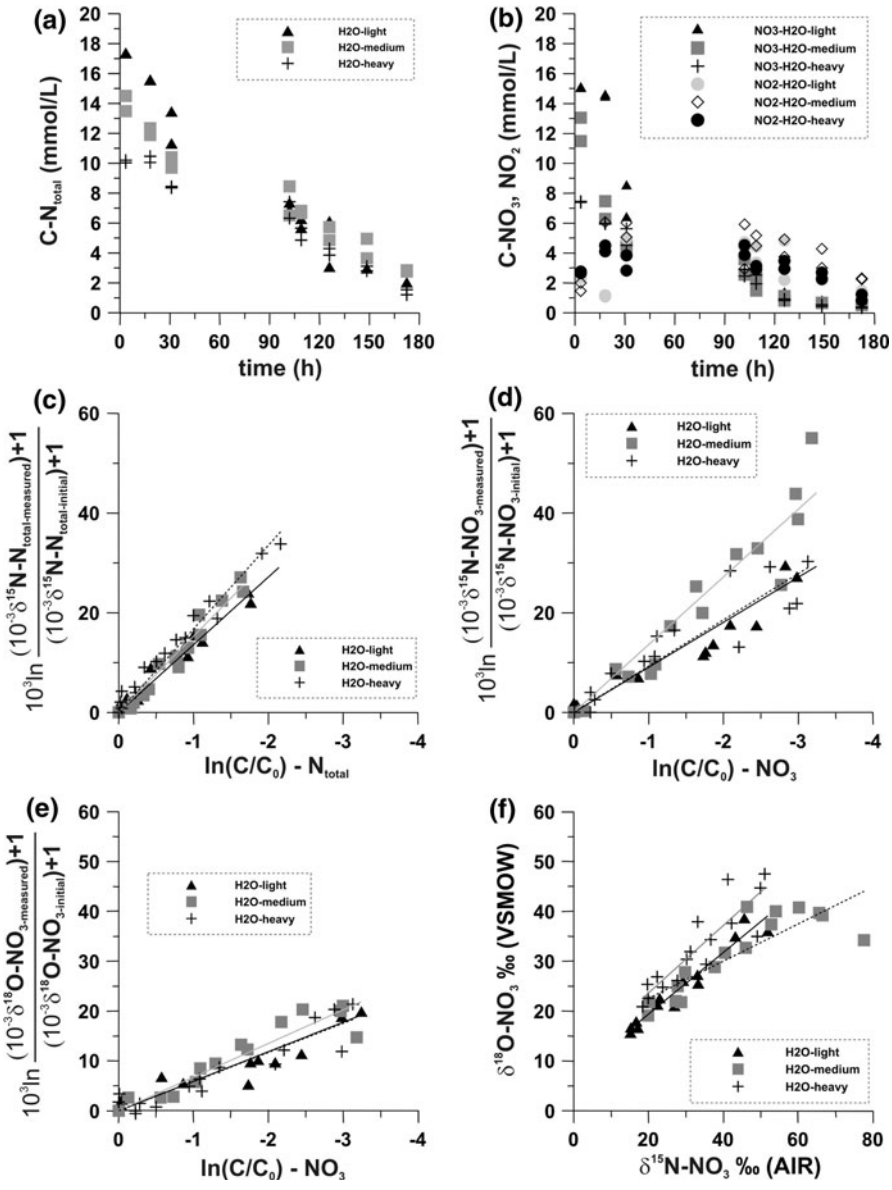

Fig. 2 Temporal development of total N (**a**), as well as nitrate and nitrite (**b**) concentrations for batch experiments with *Azoarcus* sp. using toluene as electron donor and three isotopically different waters. Furthermore, the figure shows the correlations between the residual total N pool (nitrate + nitrite) and normalized nitrogen isotope ratios (according to Eq. 2) of the total N pool (**c**), between the residual nitrate and the normalized nitrogen (**d**) and oxygen (**e**) isotope ratios of nitrate, and the between $\delta^{15}N–NO_3$ and $\delta^{18}O–NO_3$ values (**f**)

fractionation (Northrop 1981). The reaction catalysed by dissimilatory and assimilatory nitrate reductases is an oxo-transferase reaction: nitrate binds to the Mo(IV) atom in the centre of the enzyme's bis-molybdopterin guanine dinucleotide cofactor, and is reduced to nitrite, which is subsequently released (Richardson et al. 2001). The fact that nitrate reduction always seems to be linked to an isotope fractionation of the nitrogen atom indicates that the cleavage of the oxygen–nitrogen bond is a rate-limiting irreversible step in the reaction mechanism of nitrate reductases with a molybdopterin-containing cofactor.

A very important advantage of batch experiments is the presence of closed system conditions. If the experimental conditions are well constrained, no other sinks affect the remaining nitrate pool: the concentration and isotopic composition of the nitrate is exclusively determined by nitrate reduction. Therefore, batch experiments yield realistic values for $^{15}\varepsilon$ affected only by the nitrate reduction reaction, in contrast to field studies which only allow the estimation of apparent enrichment factors.

A strong enrichment of ^{15}N in the residual pool of total nitrogen or nitrate correlated with a decrease of

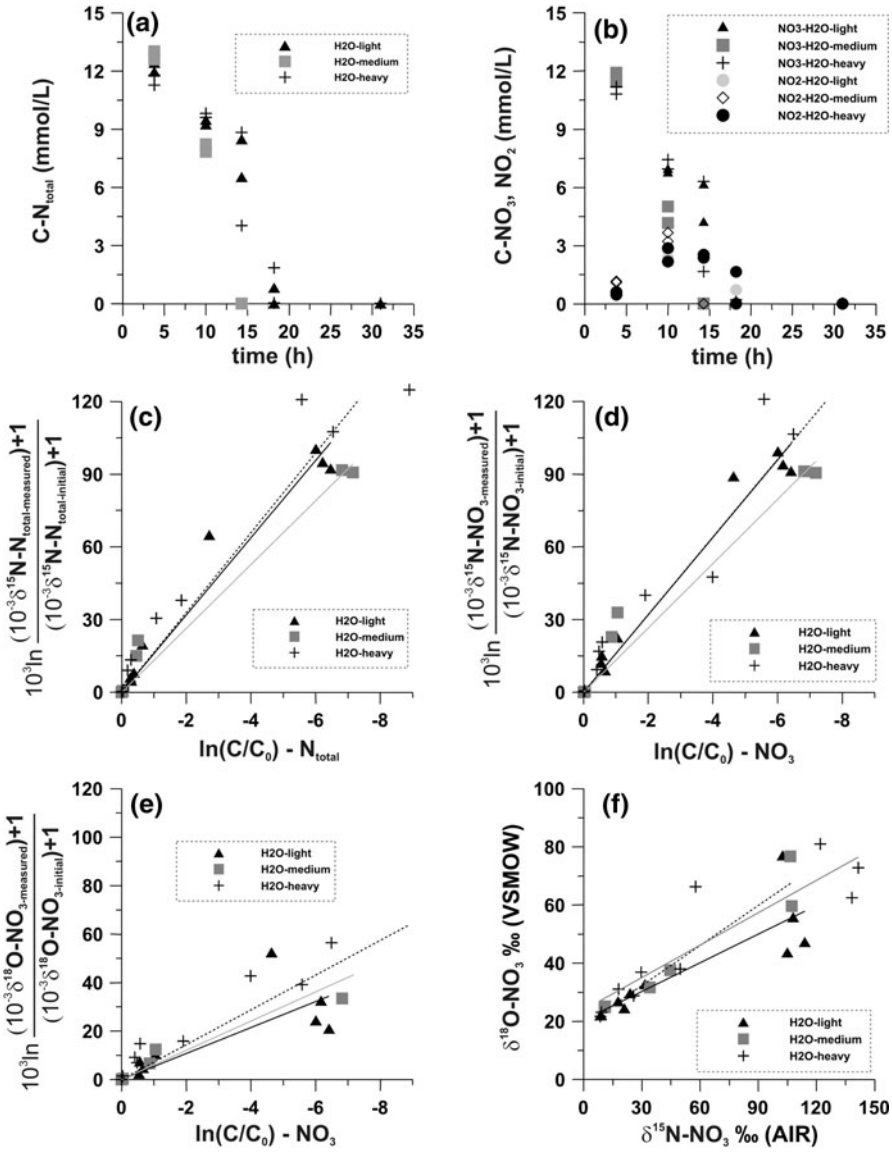

Fig. 3 Temporal development of total N (**a**), as well as nitrate and nitrite (**b**) concentrations for batch experiments with *Pseudomonas pseudoalcaligenes* F10 using succinate as electron donor and three isotopically different waters. Furthermore, the figure shows the correlations between the residual total N pool (nitrate + nitrite) and normalized nitrogen isotope ratios (according to Eq. 2) of the total N pool (**c**), between the residual nitrate and the normalized nitrogen (**d**) and oxygen (**e**) isotope ratios of nitrate, and between $\delta^{15}N$–NO_3 and $\delta^{18}O$–NO_3 values (**f**)

NO_3^- or N_{total} concentrations was found in all experiments (Figs. 1 to 3c, d). This observed relationship between the concentrations and the respective nitrogen isotope ratios is characteristic for denitrification. The most pronounced enrichment of heavy nitrogen isotopes in the remaining nitrate pool, however, occurs during the PSuc experiments where $\delta^{15}N$ values of higher that 140‰ (AIR) were obtained (Fig. 3c, d). The lowest isotope enrichment accompanied by the lowest nitrate consumption is observed for AzoTol experiments. Maximum $\delta^{15}N$–NO_3^- values are between 51 and 77‰ (AIR) for the single batches of this set of experiments (Fig. 2d). AzoSuc experiments produced maximum $\delta^{15}N$–NO_3^- values between 54 and 93‰ (AIR) (Fig. 1d). Table 1 shows the enrichment factors for total nitrogen and nitrate for all experiments, obtained by fitting the Eq. 2 to the experimental isotope data. The observed variation range is between −9.6 and −16.7‰ for the total nitrogen (bulk measurements of samples containing nitrate and nitrite) and between −8.9 and −17.2‰ for nitrate alone. Considering the summarized data presented by Lehmann et al. (2003), the nitrogen isotope enrichment factors of this study are on the lower end of $^{15}\varepsilon$ variations found in laboratory experiments and in field studies.

Table 1 Kinetic isotopic enrichment factors for total and nitrate nitrogen as well as for oxygen in denitrification batch experiments

Bacterial strain and electron donor	Isotopic type of water	$^{15}\varepsilon$ N-total (‰)	$^{15}\varepsilon$ N–NO_3^- (‰)	$^{18}\varepsilon$ NO_3^- (‰)	$^{18}\varepsilon/^{15}\varepsilon$ NO_3^-
Pseudomonas + succinate	Light	−15.5 ± 3.5	−16.2 ± 4.4	−5.5 ± 3.9	0.33
	Medium	−11.5 ± 8.3	−11.4 ± 9.9	−5.9 ± 4.9	0.49
	Heavy	−15.4 ± 4.0	−15.0 ± 4.3	−6.2 ± 2.5	0.41
Azoarcus + toluene	Light	−12.9 ± 1.5	−10.9 ± 3.1	−5.7 ± 1.7	0.52
	Medium	−16.3 ± 2.1	−14.7 ± 3.2	−6.4 ± 1.6	0.43
	Heavy	−15.3 ± 1.6	−8.6 ± 2.7	−6.8 ± 1.5	0.79
Azoarcus + succinate	Light	−9.2 ± 1.8	−10.1 ± 1.7	−4.0 ± 1.3	0.40
	Medium	−12.5 ± 3.5	−11.7 ± 2.5	−6.9 ± 2.2	0.59
	Heavy	−12.2 ± 2.0	−12.6 ± 2.5	−7.3 ± 1.7	0.57

Enrichment factors were obtained by fitting Eq. 2 to the experimental data. The uncertainties refer to the 95% confidence interval

Compared to natural systems, nitrate reduction in our experiments occurred at extremely high reaction rates. Elevated temperature, medium composition for optimal bacterial growth, as well as high nitrate and high electron donor concentrations may lead to acceleration of nitrate reduction. We cannot exclude the possibility that uptake of nitrate by the cell, transport within the cell or enzyme binding may become rate-limiting and thus lower the extent of nitrogen isotope fractionation compared to field conditions.

As a result, enrichment factors obtained during our experiments may be lower than $^{15}\varepsilon$ values observed in several field studies. This difference, however, is not likely to affect the conclusions regarding the mechanistic questions that were addressed.

In natural or experimental systems where both nitrate and nitrite are present, qualitative and quantitative information on ongoing processes may already be derived from the isotope fractionation of the total N-pool. This simple approach could save analytical resources and several time-consuming preparation steps related to the separation of nitrate and nitrite. Therefore, we compare the isotope fractionation of the total N-pool with the fractionation of the nitrate and nitrite pool.

No systematic differences are obvious between the computed enrichment factors for the isotope fractionation of the N_{total} pool with the three different strain/electron donor combinations. Even though the AzoSuc experiments yielded the lowest enrichment factors for nitrogen (−9.2 ± 1.8‰), these differences are statistically similar. The same applies for the enrichment factors characterising isotope changes of the nitrate pool. The enrichment factors for the N_{total} pool ($NO_3^- + NO_2^-$) and those of the nitrate pool are statistically identical. This is in agreement with the findings of Casciotti and McIlvin (2007) for natural water samples from the Eastern Tropical North Pacific (ETNP). However, in that case the agreement was due to the clear dominance of the nitrate content over the nitrite content so that the isotopically different nitrite did not have much influence on the isotopic composition of the total dissolved nitrogen pool. The isotopic difference between nitrate and coexisting nitrite was between 20 and 30‰ for the ETNP samples. A similar isotopic difference was observed by Böhlke et al. (2007) for coexisting nitrate and nitrite in a small freshwater stream. During our experiments, a significant isotopic difference of 25–30‰ between NO_3^- and NO_2^- was only present in the initial phase of the experiments when the nitrate concentrations were one to two orders of magnitudes higher than the nitrite concentrations (Fig. 4). In the further course of the experiments, with proceeding accumulation of dissolved nitrite, the isotopic difference decreased dramatically and sometimes disappeared completely when nitrite concentrations exceeded the concentrations of dissolved nitrate. The effect of decreasing isotopic differences between nitrate and nitrite is more pronounced in the experiments with higher reaction rates using succinate as electron donor (Fig. 4). Due to the agreement of the nitrogen isotope values of nitrate and coexisting nitrite for the majority of our experimental samples, the temporal isotopic evolution of the nitrate pool resembles very much that of the $NO_3^- + NO_2^-$ pool, so that comparable enrichment factors for nitrogen are

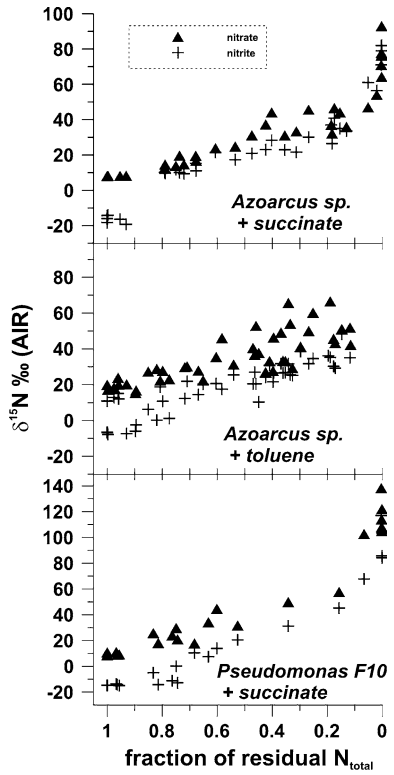

Fig. 4 Comparison of δ^{15}N-nitrate and δ^{15}N-nitrite for samples from all experiments

obtained. Nevertheless, the similarity of enrichment factors for experimental settings using different bacterial strains and different electron donors under otherwise identical circumstances suggests that the large natural variation range of $^{15}\varepsilon$, as reported by Lehmann et al. in 2003, can be attributed to certain variable environmental conditions such as nitrate supply, sediment matrix, chemical composition of the solution, pH, or temperature, assuming that dissimilatory nitrate reductases generally produce comparable $^{15}\varepsilon$ values. Whether this is actually the case remains to be proved in further isotopic studies using reference strains having different nitrate reductases.

Oxygen isotope fractionation during denitrification

It is generally accepted that both nitrate and nitrite are capable of exchanging oxygen isotopes with the ambient water (Böhlke et al. 2003; Casciotti et al. 2007). The main question remaining is whether this equilibration process or a straightforward kinetic isotope effect (as observed for nitrogen) dominates the composition of oxygen isotopes in residual nitrate during denitrification. As shown by Knöller et al. (2006) for the oxygen isotope fractionation of sulfate during bacterial sulfate reduction, a straightforward approach to investigate the influence of the isotopic composition of the ambient water on the oxygen-isotopic composition of the remaining nitrate pool is the utilization of isotopically different waters in otherwise identical experiments. The three isotopically different waters we used in our experiments (δ^{18}O between −31.2 and +4.5‰ VSMOW) cover most of the potential range for natural waters.

Assuming a dominance of an isotopic equilibration of the oxygen in the remaining nitrate pool with the oxygen of the ambient water during denitrification, as suggested by Brunner et al. in 2005, the oxygen isotope composition of nitrate should approach a steady-state value that is defined by the oxygen isotope composition of the water plus a certain equilibrium fractionation between nitrate oxygen and water oxygen. Such an oxygen isotope exchange was observed during bacterial sulfate reduction where the oxygen isotope value of the residual sulfate approaches a steady-state value that is determined by the oxygen isotope value of the ambient water (Brunner et al. 2005; Knöller et al. 2006).

Generally, the dominance of isotopic equilibration over a kinetic isotope fractionation during microbial reduction processes (Brunner et al. 2005; Knöller et al. 2006; Sturchio et al. 2007) requires firstly, intermediate reaction species that are capable of exchanging oxygen isotopes with the water rapidly, and secondly, a backward reaction from the intermediate to the reactants. Under certain conditions, depending on the initial isotope value of the reactant and on the equilibrium value with the water, the equilibration could produce isotope enrichment in the reactant pool as would be observed for a kinetic isotope effect. The equilibrium isotope fractionation for oxygen between nitrate and water is around +23 ± 1‰ for groundwater temperatures between 5 and 20°C (Böhlke et al. 2003).

A potential influence or even the dominance of an isotope equilibration over kinetic fractionation cannot necessarily be concluded from field studies. In several case studies the δ^{18}O–NO_3^- values measured are higher than those of the initial nitrate (indicating nitrate reduction) but still lower than the expected

equilibrium value with the ambient water (e.g. Böttcher et al. 1990; Lehmann et al. 2003; Casciotti and McIlvin 2007). Consequently, either kinetic fractionation or isotopic equilibration or the superposition of both processes could have produced such an isotope pattern. Very few studies report oxygen isotope values for nitrate during nitrate reduction that are higher than the expected equilibrium value with the water (e.g. Aravena and Robertson 1998; Mengis et al. 1999). For these cases, a clear dominance of the kinetic isotopic enrichment must be concluded.

Considering a potential oxygen isotope equilibration between nitrate and water and an equilibrium fractionation factor of 23‰, the final $\delta^{18}O$ values of the nitrate pool for our experiments using isotopically light, medium, and heavy water should be −8.2, 14.2, and 27.5‰ (VSMOW), respectively. This would imply a decrease of the $\delta^{18}O$ values for nitrate ($\delta^{18}O$-initial +20‰ VSMOW) during proceeding nitrate reduction in the experiments with the light and intermediate water and a slight increase during the experiments with the heavy water. The oxygen isotopic evolution of the nitrate pool would be different if the oxygen isotope exchange took place between nitrite and water followed by a reoxidation of nitrite to nitrate. In that case, an equilibrium fractionation NO_2–H_2O of 14‰ (Casciotti et al. 2007) and the incorporation of an oxygen atom from the ambient water into the newly formed nitrate molecule would determine the oxygen isotopic composition of the nitrate pool.

The actual evolution of the oxygen isotope values of nitrate with decreasing nitrate concentrations for the various experiments is shown in Figs. 1 to 3e. For all experiments a strong enrichment of heavy oxygen isotopes in the residual nitrate pool is visible, exceeding the expected isotopic equilibrium between water and nitrate by far. No significant correlation was found between the $\delta^{18}O$ of the ambient water and the maximum ^{18}O enrichment. Maximum $\delta^{18}O$ values are between 38 and 47‰, between 51 and 69‰, and between 76 and 81‰ (VSMOW) for AzoTol, AzoSuc, and PSuc experiments, respectively.

Only few enrichment factors for oxygen during denitrification have been reported (e.g. Olleros 1983; Böttcher et al. 1990; Mengis et al. 1999; Lehmann et al. 2003; Casciotti and McIlvin 2007; Granger et al. 2008) covering a range between −8 and −19‰. The oxygen isotope enrichment factors for our experiments are shown in Table 1. Generally, values for $^{18}\varepsilon$ vary between −4.2 and −7.5‰ and are thus lower than the range obtained in recent case studies. The variation of $^{18}\varepsilon$ is not systematic with respect to the bacterial strain, the utilized electron donor, or the isotopic composition of the ambient water. Apparently, the lowest enrichment factors occur in experiments with the isotopically lightest water. This would seem to suggest that a slight influence of the $\delta^{18}O$ of the water on the isotope fractionation of the nitrate oxygen during nitrate reduction might be present. However, statistical analysis revealed no significant differences between the enrichment factors obtained from the individual experiments. The isotopic composition of the ambient water does not appear to affect oxygen isotope fractionation during nitrate reduction significantly. Consequently, oxygen isotope fractionation is clearly dominated by a straightforward kinetic isotope effect.

With respect to this finding, the ratio of the enrichment factors ($^{18}\varepsilon/^{15}\varepsilon$) may be considered as a parameter representing certain reaction pathways and environmental conditions. For freshwater environments, Chen and MacQuarrie (2005) postulate a theoretical ratio of 0.51 based on reaction constants experimentally determined by Olleros (1983). Summarized field data from recent groundwater studies with a range between 0.48 and 0.67 (Chen and MacQuarrie 2005) seem to validate the theoretical value of 0.51. In contrast, the ratio of N and O enrichment factors for denitrification in marine environments is around 1 (Granger et al. 2008). The same authors assign this value to nitrate reduction by respiratory membrane-bound nitrate reductase (NAR) while smaller ratios of ca. 0.6 would be characteristic for nitrate reduction by periplasmatic nitrate reductase (NAP). However, the low significance of the NAP pathways in marine microorganisms, which is supposedly similar in terrestrial environments (Granger et al. 2008), may not be sufficient to explain the observed differences between marine and freshwater environments.

Due to the large statistical variation of the enrichment factors for nitrogen and oxygen, the $^{18}\varepsilon/^{15}\varepsilon$ ratios of our experiments cover an unusually large range between 0.33 and 0.79. This variation might be to some extent a result of the strong nitrite accumulation in the experiments; this may become partly re-oxidized during analytical procedures. The

fact that the isotopic composition of nitrate could not be measured directly but was computed from bulk measurements and pure nitrite measurements may also add uncertainty. This potential uncertainty increases considerably for high extents of consumption when nitrate and nitrite concentrations approach detection limit of the ion chromatography method. Thus, the observed variability of $^{15}\varepsilon$ and $^{18}\varepsilon$ may be related to the analytical methods, as indicated by the fact that the variability of different experiments is in the same order of magnitude as the variability observed for replicates of the same experiment. The variability within the replicates for the individual enrichment factors (95% confidence interval) is given in Table 1. Despite the scattering of data, the mean of the ratio $^{18}\varepsilon/^{15}\varepsilon$ over all our experiments is 0.50; this value confirms the theoretical value from Chen and MacQuarrie (2005). In the future, further mechanistic studies involving several bacterial strains representing different enzymatic reactions will be necessary in order to explain the puzzling difference in nitrate isotope fractionation between marine and freshwater systems.

Summary and conclusions

In batch experiments, we studied the isotope fractionation of nitrogen and oxygen during denitrification using two well-defined bacterial strains. Experimental conditions varied with respect to the electron donors (succinate and toluene) and to the isotopic composition of the ambient water (three isotopically different waters with $\delta^{18}O$ values between −31.2 and 4.5‰ VSMOW).

Knowing that the isotopic evolution of the residual nitrate-N during denitrification is controlled by a straightforward kinetic isotope effect, our intention was to examine the influence of different electron donors and of different extents of microbial nitrite accumulation on the size of the isotope effect. Our experimental results did not show any systematic correlation of the enrichment factors for nitrogen with the parameters mentioned above. The variability of $^{15}\varepsilon$ for experiments with the same bacterial strain and the same electron donor may be related to the analytical uncertainty resulting from the significant accumulation of nitrite during our experiments. Minor uncertainty may also be contributed by differences in the bacterial growth and activity under otherwise identical experimental conditions.

Furthermore, the similarity of enrichment factors for experimental settings using different bacterial strains and different electron donors under otherwise identical circumstances suggests that the large variation of $^{15}\varepsilon$ in the environment is to some extent controlled by nitrate reducing organisms, assuming that dissimilatory nitrate reductases generally produce comparable $^{15}\varepsilon$ values. However, environmental conditions such as nitrate supply, sediment matrix, and heterogeneity may also play an important role, in particular if re-oxidation of nitrite must be taken into account.

Recent investigations showed that the oxygen isotope fractionation during bacterial sulfate reduction is controlled by an isotopic equilibration with the ambient water. This, as well as the recognized potential of nitrate for exchanging oxygen isotopes with water under certain extreme environmental conditions, led us to pose the question of whether the oxygen isotope fractionation during nitrate reduction is affected by an isotopic equilibration with the oxygen of the ambient water. Our experimental results provide clear evidence that the oxygen isotope fractionation during nitrate reduction is controlled by a kinetic isotope effect. This conclusion from our experiments, however, is only valid for low-temperature processes common in aquifers. Different processes accelerating an isotope equilibration of nitrate with ambient water may be present in more extreme, high-temperature environments such as hydrothermal systems. The investigation of such high-temperature effects on the isotope fractionation during nitrate reduction is beyond the scope of this study but may be a subject of future research.

In summary, the nitrogen and oxygen isotope effect during denitrification is controlled by nitrate reduction, the first irreversible step of the denitrification pathway, whereas electron donors or equilibration reactions of nitrite with ambient water play an insignificant role. The isotope fractionation pattern of other denitrifying strains may display a different extent of isotope effects; however, if the enzyme mechanisms are similar, the fractionation will still be predominantly controlled by the first reduction step to nitrite. This may lead to characteristic $^{18}\varepsilon/^{15}\varepsilon$ ratios that can be used to analyse the nitrate reduction process in the environment. Re-oxidation of nitrite or

ammonia to nitrate may give a different isotope signature of nitrogen and oxygen isotopes of nitrate, and thus the multi-isotope analysis may be used to characterise the nitrogen cycle in aquifers.

Despite the dominance of the kinetic isotope effect for oxygen, an influence of an equilibration with the water oxygen cannot be excluded in heterogeneous aquifers when nitrite oxidation occurs. Nitrite rapidly exchanges oxygen isotope with water. Under natural conditions where the nitrite is subject to oxidation due to transport-related changes in the hydrochemical milieu, this rapid isotope exchange may indirectly affect the isotopic evolution of the nitrate pool during denitrification. The recognition of such oxidation of nitrite or nitrification processes is essential for a reliable prediction of the long-term development of the nitrate load of water resources.

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References

Aravena R, Robertson WD (1998) Use of multiple isotope tracers to evaluate denitrification in ground water: study of nitrate from a large-flux septic system plume. Ground Water 36:975–982

Böhlke JK, Denver JM (1995) Combined use of groundwater dating, chemical, and isotopic analyses to resolve the history and fate of nitrate contamination in 2 agricultural watersheds, Atlantic Coastal-Plain, Maryland. Water Resour Res 31:2319–2339

Böhlke JK, Wanty R, Tuttle M, Delin G, Landon M (2002) Denitrification in the recharge area and discharge area of a transient agricultural nitrate plume in a glacial outwash sand aquifer, Minnesota. Water Resour Res 38:1105–1131

Böhlke JK, Mroczkowski SJ, Coplen TB (2003) Oxygen isotopes in nitrate: new reference materials for O-18: O-17: O-16 measurements and observations on nitrate-water equilibration. Rapid Commun Mass Spectrom 17:1835–1846

Böhlke JK, Smith RL, Hannon JE (2007) Isotopic analysis of N and O in nitrite and nitrate by sequential selective bacterial reduction to N_2O. Anal Chem 79:5888–5895

Böttcher J, Strebel O, Voerkelius S, Schmidt HL (1990) Using isotope fractionation of nitrate nitrogen and nitrate oxygen for evaluation of microbial denitrification in a sandy aquifer. J Hydrol 114:413–424

Bozau E, Knöller K, Strauch G (2006) Nitrate degradation without N-15 enrichment: a hydrochemical and isotopic study of a fractured rock aquifer including embedded lakes. Isotopes Environ Health Stud 42:251–260

Brandes JA, Devol AH, Yoshinari T, Jayakumar DA, Naqvi SWA (1998) Isotopic composition of nitrate in the central Arabian Sea and eastern tropical North Pacific: a tracer for mixing and nitrogen cycles. Limnol Oceanogr 43:1680–1689

Brunner B, Bernasconi SM, Kleikemper J, Schroth MH (2005) A model for oxygen and sulfur isotope fractionation in sulfate during bacterial sulfate reduction processes. Geochim Cosmochim Acta 69:4773–4785

Casciotti KL, McIlvin MR (2007) Isotopic analyses of nitrate and nitrite from reference mixtures and application to Eastern Tropical North Pacific waters. Mar Chem 107:184–201

Casciotti KL, Sigman DM, Hastings MG, Böhlke JK, Hilkert A (2002) Measurement of the oxygen isotopic composition of nitrate in seawater and freshwater using the denitrifier method. Anal Chem 74:4905–4912

Casciotti KL, Böhlke JK, McIlvin MR, Mroczkowski SJ, Hannon JE (2007) Oxygen isotopes in nitrite: analysis, calibration, and equilibration. Anal Chem 79:2427–2436

Chen DJZ, MacQuarrie KTB (2005) Correlation of delta N-15 and delta O-18 in NO_3^- during denitrification in groundwater. J Environ Eng Sci 4:221–226

Chiba H, Sakai H (1985) Oxygen isotope exchange-rate between dissolved sulfate and water at hydrothermal temperatures. Geochim Cosmochim Acta 49(4):993–1000

Dolfing J, Zeyer J, Binder-Eicher P, Schwarzenbach RP (1990) Isolation and characterization of a bacterium that mineralizes toluene in the absence of molecular oxygen. Arch Microbiol 154:336–341

Einsiedl F, Mayer B (2006) Hydrodynamic and microbial processes controlling nitrate in a fissured-porous karst aquifer of the Franconian Alb, Southern Germany. Environ Sci Technol 40:6697–6702

Einsiedl F, Maloszewski P, Stichler W (2005) Estimation of denitrification potential in a karst aquifer using the N-15 and O-18 isotopes of NO_3. Biogeochemistry 72:67–86

Granger J, Sigman DM, Prokopenko MG, Lehmann MF, Tortell PD (2006) A method for nitrite removal in nitrate N and O isotope analyses. Limnol Oceanogr Methods 4:205–212

Granger J, Sigman DM, Lehmann MF, Tortell PD (2008) Nitrogen and oxygen isotope fractionation during dissimilatory nitrate reduction by denitrifying bacteria. Limnol Oceanogr 53:2533–2545

Kendall C (1998) Tracing nitrogen sources and cycling in catchments. In: Kendall C, McDonnell JJ (eds) Isotope tracers in catchment hydrology. Elsevier, Amsterdam, pp 519–576

Knöller K, Strauch G (1999) Assessment of the flow dynamic of a mining lake by stable isotope investigations. Isotopes Environ Health Stud 35:75–83

Knöller K, Vogt C, Richnow HH, Weise SM (2006) Sulfur and oxygen isotope fractionation during benzene, toluene, ethyl benzene, and xylene degradation by sulfate-reducing bacteria. Environ Sci Technol 40:3879–3885

Lehmann MF, Reichert P, Bernasconi SM, Barbieri A, McKenzie JA (2003) Modelling nitrogen and oxygen isotope fractionation during denitrification in a lacustrine redox-transition zone. Geochim Cosmochim Acta 67: 2529–2542

Lloyd RM (1968) Oxygen isotope behavior in the sulfate-water-system. J Geophys Res 73:6099–6110

Mariotti A, Germon JC, Hubert P, Kaiser P, Letolle R, Tardieux A, Tardieux P (1981) Experimental-determination of nitrogen kinetic isotope fractionation—some principles—illustration for the denitrification and nitrification processes. Plant Soil 62:413–430

Martienssen M, Schöps R (1997) Biological treatment of leachate from solid waste landfill sites—alterations in the bacterial community during the denitrification process. Water Res 31(5):1164–1170

Mengis M, Schiff SL, Harris M, English MC, Aravena R, Elgood RJ, MacLean A (1999) Multiple geochemical and isotopic approaches for assessing ground water NO_3^- elimination in a riparian zone. Ground Water 37:448–457

Moreno-Vivián C, Cabello P, Martínez-Luque M, Blasco R, Castillo F (1999) Prokaryotic nitrate reduction: molecular properties and functional distinction among bacterial nitrate reductases. J Bacteriol 181(21):6573–6584

Northrop DB (1981) The expression of isotope effects on enzyme catalyzed reactions. Annu Rev Biochem 50: 103–131

Olleros T (1983) Kinetische Isotopeneffekte der Arginase- und Nitratreduktase-Reaktion: Ein Beitrag zur Aufklärung der entsprechenden Reaktionsmechanismen. Ph.D. dissertation, Technische Universität München-Weihenstephan

Osenbrück K, Fiedler S, Knöller K, Weise SM, Sültenfuss J, Oster H, Strauch G (2006) Timescales and development of groundwater pollution by nitrate in drinking water wells of the Jahna-Aue, Saxonia, Germany. Water Resour Res 42:W12416. doi:10.1029/2006WR004977

Pfennig N, Eimhjell Ke, Jensen SL (1965) A new isolate of *Rhodospirillum fulvum* group and its photosynthetic pigments. Archiv Für Mikrobiologie 51:258–268

Rabus R, Kube M, Heider J, Beck A, Heitmann K, Widdel F, Reinhardt R (2005) The genome sequence of an anaerobic aromatic-degrading denitrifying bacterium, strain EbN1. Arch Microbiol 183:27–36

Richardson DJ, Berks BC, Russell DA, Spiro S, Taylor CJ (2001) Functional, biochemical and genetic diversity of prokaryotic nitrate reductases. Cell Mol Life Sci 58:165–178

Sigman DM, Casciotti KL, Andreani M, Barford C, Galanter M, Böhlke JK (2001) A bacterial method for the nitrogen isotopic analysis of nitrate in seawater and freshwater. Anal Chem 73:4145–4153

Song BK, Ward BB (2003) Nitrite reductase genes in halobenzoate degrading denitrifying bacteria. FEMS Microbiol Ecol 43:349–357

Sturchio NC, Böhlke JK, Beloso AD Jr, Streger SH, Heraty LJ, Hatzinger PB (2007) Oxygen and chlorine isotopic fractionation during perchlorate biodegradation: laboratory results and implications for forensics and natural attenuation studies. Environ Sci Technol 41:2796–2802

Voss M, Dippner JW, Montoya JP (2001) Nitrogen isotope patterns in the oxygen-deficient waters of the Eastern Tropical North Pacific Ocean. Deep Sea Res I 48:1905–1921

Widdel F, Rabus R (2001) Anaerobic biodegradation of saturated and aromatic hydrocarbons. Curr Opin Biotechnol 12:259–276

Zumft WG (1997) Cell biology and molecular basis of denitrification. Microbiol Mol Biol Rev 61:533–616